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ROLE OF ION CHANNELS IN PROGRAMMED CELL DEATH INDUCED BY HYPEROSMOTIC STRESSES IN PLANT CELLS

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Abstract

The work presented in the present thesis relates to the role of ion channels in response to (ionic and non-ionic) hyperosmotic stresses and their interactions with signaling events leading to PCD in plant. Early cell responses such as cytosolic calcium increase and ROS production classically involved in PCD process, seems not to be involved in hyperosmotic-induced cell death in BY2 tobacco and *A. thaliana* cultured cells. When BY2 tobacco cells were subjected to hyperosmotic stress, an early influx of sodium through non-selective cation channels participates in the development of PCD through mitochondrial dysfunction and NADPH-oxidase-dependent $O_2^{\cdot -}$ generation. On the contrary, non-ionic hyperosmotic stress resulted in an early decrease in anion currents. To further investigate the role of anion channels in non-ionic hyperosmotic stress further experiments were conducted by using *A.thaliana* cells of the anion channel mutant SLAC1. Results showed that the delayed activation of SLAC1 channels was involved in the non-ionic hyperosmotic stress induced pathway leading to cell death. Interestingly, the early anion channel activity decrease could participate to signalisation or osmotic adjustment allowing cell adaptation and survival, when a second set of events, namely superoxide anion ($O_2^{\bullet -}$) generation by NADPH-oxidase and anion channel activation could participate in PCD development of a part of the cell population. In addition, the potential role of small peptides belonging to the FMRFamide-like peptide (FLP) family described in metazoan in osmoregulation in *A. thaliana* was investigated. By using synthetic peptides, based on FLPs homolog genes existing in *A. thaliana*, it was possible to demonstrate that these putative FLPs are involved in hyperosmotic stress response. Overall, the present work shed light on the importance and the complexity of ion channels regulation in the signaling pathways and the processes leading to PCD.

Résumé

Le travaux présenté dans cette thèse concerne le rôle des canaux ioniques de la membrane plasmique en réponse à des stress salins et non salins ainsi qu'aux interactions possibles avec d'autres événements de signalisation conduisant à la mort cellulaire programmée (PCD). Nous avons montré que les réponses cellulaires précoces:

tels que l`augmentation du calcium cytosolique et la production de ROS, classiquement impliqués lors de la PCD, ne semblaient pas être impliqué dans la mort cellulaire induite par les stress hyperosmotiques chez les cellules en culture de tabacco BY2 ou d'*A. thaliana*. Nous avons montré que, dans les cas de stress salin chez les cellules de BY2 un influx précoce de sodium à travers des canaux cationiques non spécifiques participe au développement de la PCD en entraînant un disfonctionement mitochondrial et la production de $O_2^{\bullet -}$ par des NADPH oxydases. Dans le cas de stress hyperosmotique non-ionique, nous avons observé une diminaution précoce de l'intensité des courants anioniques. Afin de poursuivre l'étude du rôle des canaux anioniques lors du stress hyperosmotique non salin, nous avons utilisé des cellules *A.thaliana* nous permettant de travailler avec le mutant de canal anionique SLAC1. Nous avons constaté que l'activation retardée des canaux SLAC1 participait au développement de la PCD induite par un stress hyperosmotique non salin. La réduction précoce de l'activité des canaux anioniques pourrait participer à la signalisation ou l'ajustement osmotique permettant l'adaptation et la survie cellulaire alors que des évènements retardés, à savoir la production d'anion superoxyde ($O_2^{\bullet -}$) par les NADPH-oxydases et l'activation des canaux anioniques pourraient participer au développement de la PCD d'une partie de la population cellulaire. Nous avons aussi étudié le rôle potentiel des petits peptides appartenant à la famille des peptides FMRFamide décrite chez les métazoaires à l'osmorégulation chez des cellules d'*A. thaliana*. Des génes susceptibles de coder de tels peptides sont en effet présent dans le génome d'*A. thaliana*. En utilisant des peptides synthétiques, nous avons montré que ces FLP_S putatifs pourraient participer aux réponses induites losr de stress hyperosmotique chez les plantes. Ce travail illustre la complexité et l'importance de la régulation des canaux ioniques dans les voies de signalisation et les processus conduisant à la PCD.

Riassunto

Il lavoro presentato in questa tesi riguarda il ruolo dei canali ionici nella membrana plasmatica in risposta a stress di tipo iperosmotico (salino e non salino) e il loro coinvolgimento nelle vie di segnalazione che portano alla PCD in pianta. Le risposte precoci (es. aumento di calcio citosolico e produzione di ROS) a stress di tipo iperosmotico, coinvolte negli eventi che portano alla PCD, non sembrano essere

implicate nel modello da noi studiato (cellule di tabacco BY2 e di *Arabidopsis*). Abbiamo dimostrato che nel caso di stress ionico l'influsso di sodio attraverso i canali NSCC (Non Selective Cation Channels) partecipa allo sviluppo della PCD inducendo depolarizzazione mitocondriale e produzione di $O_2^{\cdot -}$ da parte dell'enzima NADPH ossidasi. Nel caso di stress non ionico abbiamo riscontrato una precoce diminuzione delle correnti anioniche. Per studiare meglio il ruolo dei canali anionici abbiamo usato cellule di *A.thaliana* che ci permettono di lavorare su mutanti. Abbiamo dimostrato che i canali SLAC1 risultano coinvolti negli eventi che portano alla PCD in seguito a stress di tipo non ionico. La diminuzione di correnti anioniche potrebbe partecipare alla regolazione osmotica che permette l'adattamento cellulare. Un'attivazione delle correnti anioniche e generazione di $O_2^{\cdot -}$ da parte dell'enzima NADPH-ossidasi potrebbe partecipare allo sviluppo della PCD di una parte della popolazione cellulare. Abbiamo inoltre indagato il potenziale ruolo di piccoli peptidi appartenenti alla famiglia FMRF amide-like peptide (FLP) nella regolazione di stress osmotico in colture cellulari di *A.thaliana*. Utilizzando peptidi sintetici, abbiamo dimostrato che questi peptidi potrebbero partecipare alla regolazione di stress osmotico nelle piante. Questo lavoro mette in luce l'importanza e la complessità della regolazione dei canali ionici nelle vie di segnalazione degli eventi che portano alla PCD.

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Chapter 1

Introduction

1 Hyperomotic stress

In nature, plants are subjected to different biotic and abiotic stresses. Among abiotic stresses, drought and salinity are the primary causes of crop loss worldwide. Global warming, due to climate change, will enhance these environmental stresses that may severely affect crop productivity (Peters *et al.*, 2013). Drought, defined as soil and/or atmospheric water deficit, reduces plants water potential and turgor. Among drought effects are loss of water, which is involved in stomatal closure and limitation of gas exchange that results in reduced plant growth and productivity and could lead to death. It is expected an increase of drought frequency mainly due to climate change, as a result of decreasing regional precipitations and increasing evaporation driven by global warming (Seneviratne *et al.*, 2012; Sheffield *et al.*, 2012). Since 1970 significant increases in drought extent and severity have already been estimated for Africa, southern Europe, east and south Asia and eastern Australia (Lobell and Gourdji, 2012; Sheffield and Wood, 2008). Water deficit and aridity conditions lead also to salinization of the soil. Salinity has also been identified as one of the major threats because of its degrading effects on landscapes (Ghassemi *et al.*, 1995). Increased salinization of arable lands is expected, resulting in 50% land loss by the year 2050 (Wang *et al.*, 2003).

Drought and salinity have osmotic, ionic and nutritional constraint effects on plants. These effects lead to growth retardation, metabolic disturbances and oxidative stress. Plants may tolerate and adapt to these stressors with different mechanisms including changed leaf architecture, osmotic adjustment, ion exclusion and compartmentalization and a more efficient reactive oxygen species (ROS) scavenging systems. However, depending on the strength and duration of these stresses and on the plant's genetic features, it can be a matter of life or death since there is always the so-called "stability limit", which when exceeded leads the organism to death. In Figure 1 is shown the main abiotic stress that affects plants in nature.

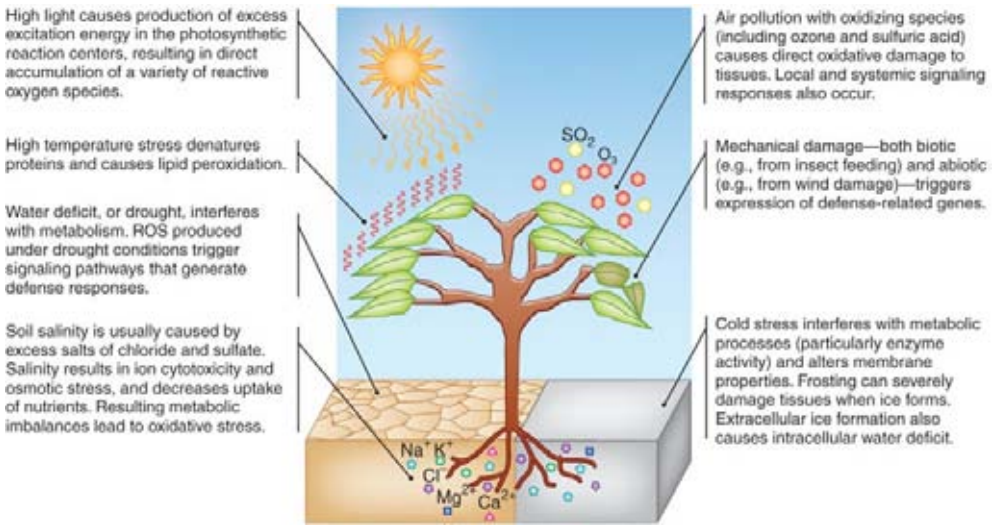

Figure 1: Overview of abiotic stresses that affect plants (Vickers *et al.*, 2009)

1.2 General plant responses to osmotic stresses

Plants have evolved sophisticated systems to detect and respond to osmotic stresses (Hirayama and Shinozaki, 2010). The general plant osmotic stresses response include a complex signaling pathway, that involves: (i) the reception of the stress, through the osmosensors that are probably located at the plasma membrane level; (ii) the signal transdution (i.e. osmosignaling) to the nucleus where (iii) the last step of controlled expression of specific genes occurs. The molecular basis of osmosensing is not fully understood in plants and little is known about the receptors that detect osmotic stress, much information is available for numerous transcription factors acting in the regulation of gene expression. The signals transductions, amplify and integrate diverse external signals to generate responses such as changes in enzyme activity, gene expression or ion-channel activity.

1.2.1 Osmosensor

Osmosensing is pivotal for mantaining a normal water status and all cellular functions in plants. All living cells developed sophisticated tools to sense and respond quickly to osmotic stresses. Osmosensors are proteins whose primary role is to monitor fluctuations in external osmolarity and start an activation of signalling pathways for osmo-adaptation. (Osakabe *et al.*, 2011; Yamaguchi-Shinozaki and Shinozaki, 2006). In general, an osmosensor can be defined as a component that is altered by osmotic stresses. The molecular basis of osmosensing in plants is not fully understood and little is known about the receptors that detect osmotic stress. Signals emanate from several osmosensors are involved in a combinational way that allows highly specific outcomes despite the lack of exclusively specific osmosensors (Kültz, 2012). It has been hypothesized that osmosensing involve the sensing of cell volume, shape, membrane tension or macromolecular crowding by osmosensor proteins (Burg *et al.*, 2007; Kumar *et al.*, 2013; Schliess *et al.*, 2007). In animal cells slight osmolality can be sensed by calcium sensing receptors and transient receptor potential (TRP), in particulary TRPV4 in vertebrates (Liedtke *et al.*, 2000; Nilius *et al.*, 2004; Strotmann *et al.*, 2000; Wissenbach *et al.*, 2000). Although is not very clear whether TRP is involved in volume regulation, it was found that it mediates the Ca^{2+} increase which subsequently stimulates the Regulatory Volume Decrease (RVD) with the activation of K^+ channels (Numata *et al.*, 2007). Interestingly, a recent study made a parallelism between plant and animal kingdom after finding a Calcium permeable Stress-gated cation Channel 1 (AtCSC1) in *A. thaliana* (Hou *et al.*, 2014). The analysis of whole-cell two-electrode voltage clamp (TEVC) on *Xenopus* oocytes expressing this gene, led to the conclusion that this protein is a ion channel, which is permeable to various cations, including Ca^{2+},K^+ and Na^+. Interestingly, the C-terminal of AtCSC1 comprising domain of unknown function 221 (DUF221) belongs to transmembrane channel-like (TMC) proteins that are cation channel components. Since CSCs are a family of cation channels permeable to calcium triggered by physical signals such as hyperosmotic stress, and these properties are reminiscent of TRPs in animals, these channels are candidates for involvement in osmo- or mechano-sensitive calcium signaling processes in plants (Hou *et al.*, 2014).

Receptor-like kinases (RLKs), which form a large gene family in plants, contain Ser/Thr kinase as a cytosolic domain while having structural elements similar to animal

receptor tyrosine kinases (RTKs) (Osakabe *et al.*, 2013; Shiu and Bleecker, 2001). The RLKs are involved in water stress responses, and this receptor like kinase (RLK) family is composed by more than 600 members, with the leucine rich-repeat (LRR)-RLKs forming the largest subgroup (Gish and Clark, 2011; Osakabe *et al.*, 2011). Several RLKs localized in the plasma membrane are known to be involved in the early steps of osmotic-stress signaling in a variety of plant species (Osakabe *et al.*, 2013). Several studies have been performed to explore the involvement of RLK gene in environmental stresses (Chae *et al.*, 2009; Lehti-Shiu *et al.*, 2009). RLKs involvement in sensing environmental cues and abiotic stresses have been reported for various plant species, such as Arabidopsis (Bai *et al.*, 2009; Osakabe *et al.*, 2005; Osakabe *et al.*, 2010; Tanaka *et al.*, 2012), rice (Oryza sativa) (Ouyang *et al.*, 2010), *Medicago truncatula* (De Lorenzo *et al.*, 2009), and soja (Yang *et al.*, 2010). In particular in plants there are some RLKs such as RPK1, CYSTEINE-RICH RLK (CRK36), PROLINE-RICH-EXTENSIN-LIKE RLK4 (PERK4), and GHR1 (GUARD CELL HYDROGEN PEROXIDE-RESISTANT1), that studies have been reported to control water stress signalling directly in Arabidopsis (Bai *et al.*, 2009; Osakabe *et al.*, 2005; Osakabe *et al.*, 2010; Osakabe *et al.*, 2013). Interestingly, de Lorenzo et al. (2009) identified a salt stress-inducible LRR-RLK gene (SRLK) in M. truncatula, and root growth inhibition by high salinity stress was reduced in SRLK RNA interference (RNAi) transgenic Medicago roots. Epidermal cell-specific expression of the SRLK gene was observed in roots under salt stress, and SRLK was shown to control the expression level of several salt-responsive genes. These findings suggest that SRLK activates the signalling pathway involved in the adaptive response of Medicago roots to salt stress (De Lorenzo *et al.*, 2009; Osakabe *et al.*, 2013). It tempting to suppose that plants as well as vertebrate cells, have evolved a sophisticate mechanism at the plasma membrane to sense osmotic changes. Clearly, it is not surprising that plants have evolved mechanisms to sense osmotic forces at the plasma membrane, as these are the first cell component to come in contact with the stress. In animal cells, because they lack a cell wall, as well as the membrane itself cannot provide a mechanical barrier, the actin cytoskeleton has been proposed to control shape after osmotic challenge in animal cells (Papakonstanti *et al.*, 2001). In different cell lines, a rapid organization of the actin filaments of cytoskeleton has been observed as a result of osmotic stress. This type of organization

varies between different cell types, but generally leads to an increase of the F-actin (Pedersen *et al.*, 1999). Recent studies have demonstrated that microtubule-associated (MT) to the cytoskeleton are regulated by osmotic shrinkage. Nevertheless, the mechanisms that link the sensing of the perturbation of the volume to the rearrangement of the cytoskeleton have not been completely elucidated. In plants there is a strong and transient response of microtubules to hyperosmotic stress: microtubules first disappear, but soon they are replaced by massive bundles, called macrotubules (Komis *et al.*, 2002). The formation of macrotubules can be suppressed by oryzalin, which at the same time blocks osmoadaptation, demonstrating that this microtubule response is not a product of adaptation but represents an essential event. A pharmacological study on this phenomenon revealed that inhibitors of phospholipase D, such as *n*-butanol or *N*-acetylethanolamine suppress both macrotubule formation and osmotic adaptation (Komis *et al.*, 2006). In vertebrate cells, the integrins have an important role in osmotic sensing. Different studies had shown (Komis *et al.*, 2002) that integrins were activated as a result of disruption of the cell volume, and has been proposed that they serve as the volume sensors after swelling or shrinkage. It was demonstrated an involvement of integrins in the regulation of osmosis and in the movement of ions across cell membranes, in particular they control the flow of calcium, potassium and chlorine, either directly (Hoffmann and Pedersen, 2011) or indirectly (Hoffmann and Pedersen, 2011; Shakibaei and Mobasheri, 2003). It is difficult to transfer these mechanisms to plants, since they have not yet identified canonical integrins in plant cells (Baluška *et al.*, 2003) and the situation in plant cells differs fundamentally. In plants, the shape is maintained thanks the cell wall, where elongate load-bearing elements (cellulose microfibrils) are embedded in an amorphous matrix (hemicelluloses, pectins, proteins). In these cells, the interphasic plant cytoskeleton is not directly required to support cell shape and therefore it is free to adopt additional functions. In plants, cortical microtubules are connected with the extracellular matrix through transmembrane proteins that have not been identified so far, but it seems that they share some analogies with animal integrins (Baluška *et al.*, 2003). This link seems to stabilize cortical microtubules. For instance, removal of the cell wall makes microtubules more cold-sensitive in tobacco cells (Akashi *et al.*, 1990). Moreover, cobtorin, a compound identified from a screen that specifically perturbs the parallelity of microtubules and

microfibrils (Yoneda *et al.*, 2007) has meanwhile been found to target the cell wall pectins.

1.3 Signalisation in response to osmotic stress

Reactive Oxygen Species (ROS), Calcium (Ca^{2+}), Mitogen activated protein kinases (MAPK) and ion channels are recognized to be important in plant signalisation responses to osmotic stress.

1.3.1 Reactive Oxygen Species (ROS) signalling

The ROS signalling network controls a wide range of biological processes such as growth, development and responses to biotic and/or abiotic stimuli (Mittler *et al.*, 2011). ROS are produced as a normal cellular process in all aerobic organisms and include free radicals such as superoxide anion (O_2^-) hydroxyl radical ($^{\bullet}OH$), as well as non-radical molecules like hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). ROS are produced by reduction of molecular oxygen (O_2) by means of the electron transport activities of chloroplasts, mitochondria, and plasma membranes (Foyer *et al.*, 1995; Heyno *et al.*, 2011). Also NADPH oxidases respiratory burst oxidase homologues (RBOHs), is involved in ROS production (Suzuki *et al.*, 2011; Torres and Dangl, 2005). During osmotic stress, drought and salinity, ROS production increases (Serrato *et al.*, 2004). During water deficit there is an accumulation of abscissic acid (ABA) in leaves, which minimize water loss by stimulating stomatal guard cell closure (Li *et al.*, 2000). The CO_2 available for photosynthesis is reduced and it can misdirect electrons in the photosystem and lead to the formation of ROS. Unlike biotic stress, where the so called oxidative burst is frequently involved in the events that lead to programmed cell death (PCD), the role of ROS production during osmotic stress needs to be demonstrated. Under abiotic stress ROS seem to be involved in a dual process depending on their cellular concentrations. At low levels ROS have functions as components of a pathway that lead to stress defense/acclimation responses (Vranova *et al.*, 2002). When reaching a level of phytotoxicity ROS become detrimental and participate in the events triggering to cell death (Mittler, 2002).

1.3.1.1 Plant ROS generation

1.3.1.1.1 Singlet Oxygen ($^1O_2$)

Singlet oxygen ($^1O_2$) is a highly reactive short-lived product (half-life, approximately 200 ns) with a strong oxidizing potential (Apel and Hirt, 2004; Vellosillo *et al.*, 2010). $^1O_2$ is produced constitutively by a photochemical excitation of the chlorophyll of photosystem II with oxygen (Krieger-Liszkay *et al.*, 2008). In Figure 2 is shown the generation of $^1O_2$ by excited chlorophyll (Fischer *et al.*,2013). $^1O_2$ can be also produced by phytoxins during plant-pathogen interaction. and by peroxidases (POX) (Kanofsky, 2000; Kawano *et al.*, 1998).

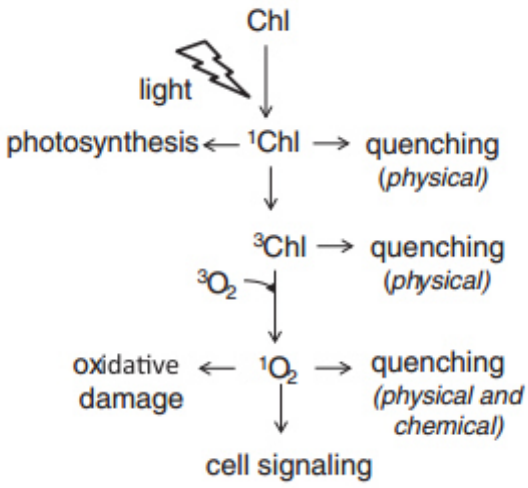

Figure 2: $^1O_2$ generation by excited chlorophyll (from Fischer *et al.*,2013)

Peroxidases (POXs) could also be involved in $^1O_2$ generation (Kanofsky *et al.*, 1988; Kawano *et al.*, 1998). Peroxidases are involved in a wide range of physiological processes, such as lignification, suberization, auxin catabolism, wound healing and defense mechanisms against pathogen infection (Kawano, 2003). The plant POXs in the apoplastic space are often involved in ROS generation. Peroxidases are considered essential to catalyze the generation of aromatic oxyl radicals from several aromatic compounds (Kawano, 2003; Takahama and Yoshitama, 1998) and this POX-dependent production is often associated to ROS generation (Kawano, 2003).

1.3.1.1.2 Superoxide anion ($O_2^{\cdot -}$)

The first reaction of molecular oxygen is a reduction to form thesuperoxide anion ($O_2^{\cdot -}$).

$$O_2 + e^- \rightarrow O_2^{\cdot -}$$

$O_2^{\cdot -}$ is the product of the reduction of oxygen by one electron. An enzymatic sources include NAD(P)H oxidases located on the cell plasma membrane of peroxisomes. Dismutation of $O_2^{\cdot -}$ produces hydrogen peroxide (H_2O_2), which may be reduced to water (H_2O) or partially reduced to hydroxyl radical ($OH^{\bullet}$).

The partial reduction of O_2 may generate different Reactive Oxygen Species (ROS).Moreover, ROS may be produced through transition metal-mediated pathways in the Fenton reaction or in the net Haber-Weiss reactions. A schematic representation of the sequential ROS production is shown in Figure 3

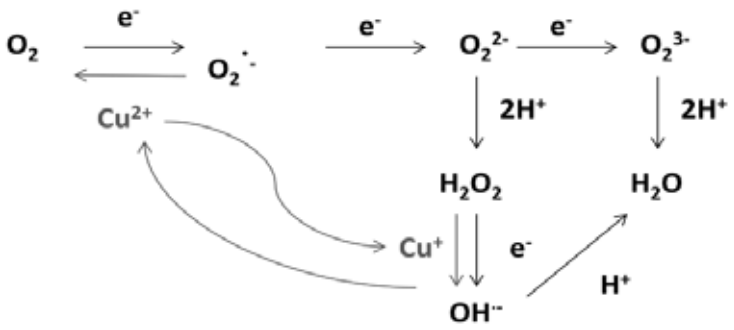

Figure 3: ROS production by multistep reduction of oxygen.(from Appel & Hirt, 2004; Gechev *et al.*,2006). Grey lines show the Haber-Weiss reactions.

1.3.1.1.3 Hydrogen peroxide (H_2O_2)

The hydrogen peroxide production represents an evolutionary strategy performed by plants. In response to various environmental stimuli there is an increase of O_2 production, which is subsequently converted to hydrogen peroxide (H_2O_2).

As the $O_2^{\bullet-}$ is relatively unstable, it can be converted to O_2 or in reaction with a proton to H_2O_2, spontaneously or catalyzed by the enzyme superoxide dismutase (SOD):

$$O_2 + 2H^+ + 2e^- \rightarrow H_2O_2$$

H_2O_2 is a relatively long-life ROS, it can pass through the cellular membranes and act as signalling molecule in cell-to-cell communication because it's diffusable between cells (Bienert *et al.*, 2006).It has been shown that ROS participate in various types of cell-to-cell communication in animals and plants. For example in animal cells ROS regulate the formation of intracellular gap junctions and signal transduction (Upham and Trosko, 2009) involved in apoptosis in adjacent animal cells (Widel, 2012).

1.3.1.1.4 Hydroxyl radical ($HO^\bullet$)

The hydroxyl radical ($HO^\bullet$), one of the strongest oxidant, is highly reactive and interact with proteins, nucleic acids, lipids and phospholipids. $OH^\bullet$ is involved in radical reactions and then is the major responsible for irreversible changes of macromolecules and damages to organelles. $OH^\bullet$ has a very short time-life (10^{-9}s) and interacts exclusively in the environment surrounding the site of production.

H_2O_2 takes part in the formation of $HO^\bullet$, which is produced in the reaction between H_2O_2 and Fe^{2+} acting as a metal catalyst(Fenton reaction). The equation below represents the main way of $HO^\bullet$ production:

$$O_2^{\bullet-} + Fe^{3+} \rightarrow Fe^{2+} + O_2$$
$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + HO^\bullet + HO^- \text{ (Fenton reaction)}$$
$$O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + HO^\bullet + HO^- \text{ (Haber-Weiss reaction)}$$

1.3.1.1.5 Nitric oxide ($NO^\bullet$)

Nitric oxide is a radical synthesized from arginine by NO synthase (NOS) in mammalian cells (Wendehenne *et al.*, 2001). In plants the system responsible for its synthesis is unknown yet. Its derivate peroxynitrite $ONOO^-$ can react with O_2^- and induce protein modification by tyrosine nitration(Gaupels *et al.*, 2011).Altough the

peroxynitrite is highly reactive and toxic in animals, it is not involved in NO-mediated cell death in plants (Delledonne *et al.*, 1998; Gaupels *et al.*, 2011).

1.3.1.2 Sources of ROS in plant cells

As mentioned above, ROS are produced by plant cells under normal conditions as products of metabolic processes and as responses to various environmental stresses. Different plant cell compartments are involved in ROS production. Chloroplasts, mitochondria and peroxisomes are considered the main ROS producers during abiotic stresses(Asada, 2006b; Bose *et al.*, 2013; Mittler, 2002; Mittler *et al.*, 2004). A schematic representation of ROS production in plant cell compartments is shown in Figure 4.

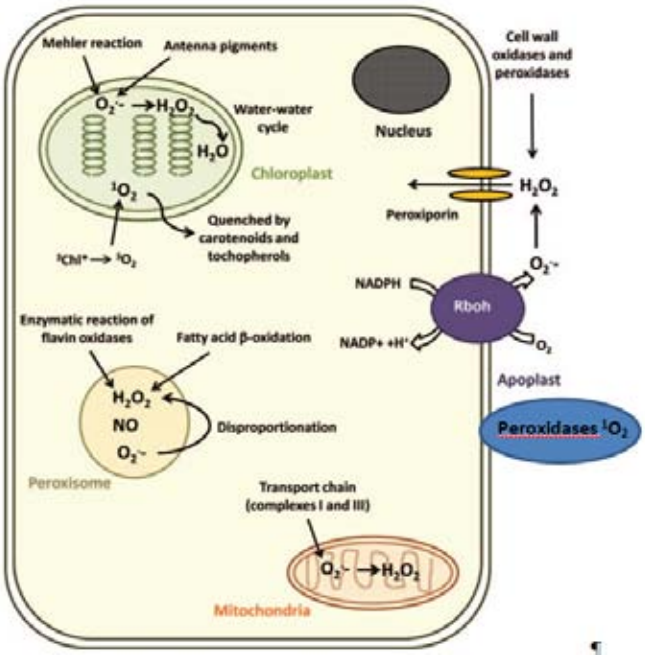

Figure 4:Sources of ROS production in plant cells (Adapted from Bose *et al*.,2013)

1.3.1.2.1 Chloroplasts

In the light chloroplasts produce ROS through photosystem I (PSI) and PSII electron transport chain (ECT) that are the main reactions centres (Asada, 2006a; Foyer and Noctor, 2003). The antenna pigments are the main producers of $O_2^{\cdot-}$by the Mehler

reaction at PSI (Asada,2006). After, H_2O_2 is produced spontaneously or by a reaction catalyzed by SOD and then further reduced by the action of ascorbate peroxidases (APXs) in the water–water cycle (Asada, 2006). In addition singlet oxygen $^1O_2$ can be produced during photosynthesis (Laloi *et al.*, 2004). It was shown an enhanced $^1O_2$ generation at PSI during water stress (Miller *et al.*, 2010). The reduced CO_2 availability due to stomata closure and the excessive exposure to light are involved in this enhanced $^1O_2$ generation.

1.3.1.2.2 Peroxisomes

The peroxisomes are a type of microbody with a single membrane. Peroxisomes are intracellular organelles mediating a wide variety of biosynthetic and biodegradative reactions. These organelles generate large quantities of ROS (Nyathi and Baker, 2006). Peroxisomes are also involved in senescence (Rio *et al.*, 2006) and heavy metal toxicity (Romero-Puertas *et al.*, 1999). Peroxisomes can be divided into three groups: glyoxysomes, leaf peroxisomes and not specialized peroxisomes. Glyoxysomes (specialized peroxisomes) are storage organelles involved in lipid mobilization. Like mitochondria and chloroplasts, peroxisomes produce radicals $O_2^{\cdot-}$ due to their metabolism. There are two O_2^- generation sites in peroxisomes, in the organelles and in peroxisomal membranes. In the organelles the xanthine oxidase (XOD) catalyzes the oxidation of xanthine and hypoxanthine to uric acid. In the peroxisomal membranes, an electron transport chain composed by NADH and cytochrome b generates $O_2^{\cdot-}$. Also the monodehydroascorbate reductase (MDHAR) generates the $O_2^{\cdot-}$. The H_2O_2 is produced during different processes, including the photorespiration glycolate oxidase reaction, fatty acids β-oxidation and disproportionation of $O_2^{\cdot-}$(Foyer and Noctor, 2009). Peroxisomes can also produce nitric oxide (Corpas *et al.*, 2011). Peroxisomes seem to be involved in ROS dependent response to drought. Riviero et al. (2007) have shown that transegenic tobacco plants expressing the isopentenyltransferase (IPT) gene, encoding an enzyme that catalyzes the rate-limiting step in cytokinin (CKs) biosynthesis, under control of a drought-induced senescence-associated-receptor-protein-kinase (SARK) promoter, had an enhanced drought tolerance (Miller *et al.*, 2010; Rivero *et al.*, 2007). In SARK-IPT transgenic plants there is an increase of

modulation of ROS metabolism genes of peroxisomal ascorbate–glutathione (AsA–GSH) cycle genes (Miller *et al.*, 2010).

1.3.1.2.3 Mitochondria

In plants mitochondria, complexes I and III are the main sites of ROS production and they are overall the main sites of ROS production (Møller, 2001), especially in non-green tissues as roots, where they are considered as the main source of ROS generation (Bose *et al.*, 2013)B. The enzyme alternative oxidase (AOX), contributes to H_2O_2 generation (Apel and Hirt, 2004). Moreover, mitochondria are involved in NO generation due to the reduction of nitrite via a nitrite reductase that has been shown to be dependent on the mitochondrial electron transport (Planchet *et al.*, 2005). It is known that the mitochondrial ROS production enhances during abiotic stresses, especially drought and salinity (Pastore *et al.*, 2007). During drought stress the respiration rate increases to compensate the reduced rate of chloroplast ATP synthesis, consequently ROS production in mitochondria is enhanced.

1.3.1.2.4 Apoplast

The apoplast represents an important site for H_2O_2 production in response to various abiotic stresses, such as drought and salinity (Hernandez *et al.*, 2001; Jubany-Mari *et al.*, 2009).

Their production depends on several groups of enzymes, including cell wall peroxidases and plasma membrane NADPH oxidases (e.g.AtRbohD and AtRbohF encode two major NADPH oxidases expressed in guard and mesophyll cells in Arabidopsis), that generate $O_2^{\cdot-}$ by oxidizing NADPH and transferring the electron to oxygen (O_2) (Sagi and Fluhr, 2006).This ROS apoplastic generation is also known as “oxidative burst” during the hypersensitive responses under a pathogen attack and also regulate cell growth, development, and programmed cell death (PCD) (Foreman *et al.*, 2003; Gapper and Dolan, 2006),(Laloi *et al.*, 2007; Sagi and Fluhr, 2006; Torres *et al.*, 2002). During drought and salt stress, accumulation of H_2O_2 could be involved in acclimation responses of plant to drought and salt stresses. It was shown that the reduction of stress-induced apoplastic ROS formation is associated with a decrease in leaf elongation. This response was observed after NaCl but not osmotic treatments. It was found that the

apoplast is a presumed site of singlet oxygen $^1O_2$ procuction by apoplastic peroxidases (POXs) (Kawano *et al.*, 1998).

1.3.1.3 ROS scavenging during osmotic stress

As mentioned above, ROS are continuously produced as metabolic products of various pathways localized in different cellular compartments (Foyer and Noctor, 2013). Due to their reactivity, toxicity and involvement in different processes such as growth, development and programmed cell death, plants have evolved complex different ROS scavenging mechanisms that mainly consist in enzymatic and non-enzymatic pathways to control ROS levels. In plant cells, specific ROS producing and scavenging systems were found in different organelles, such as chloroplasts, mitochondria and peroxisomes. ROS-scavenging pathways from different cellular compartments are coordinated with each other (Pang and Wang, 2008).

3.1.3.1 Enzymatic control of ROS level

The antioxidative defense system comprises several enzymes such as: superoxide dismutase (SOD),catalase (CAT), guaiacol peroxidase (GPX), enzymes of ascorbate-glutathione (AsA-GSH) cycle: ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) (Foyer and Noctor, 2013). Figure 5 shows the localization of ROS generation and scavenging pathways.

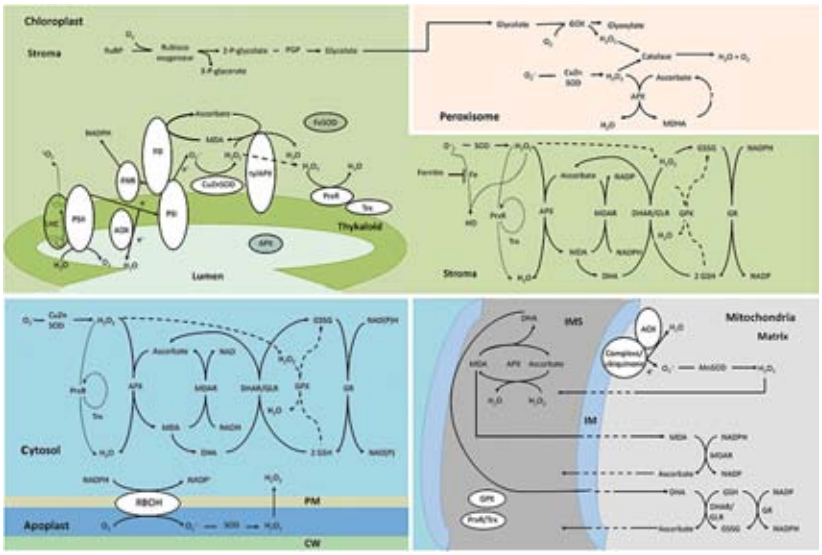

Figure 5: Localization of reactive oxygen species (ROS) generation and scavenging pathways in plant cells. The water–water cycle detoxifies $O_2^{\cdot -}$ and H_2O_2 and alternative oxidase (AOX; Immutans) reduces the production rate of $O_2^{\cdot -}$in thylakoids [top left; in some plants iron superoxide dismutase (FeSOD) might replace CuZnSOD in the chloroplast]. ROS that escape this cycle and/or are produced in the stroma undergo detoxification by SOD and the stromal ascorbate–glutathione cycle. Peroxiredoxin (PrxR) and glutathione peroxidase (GPX) are also involved in H_2O_2 removal in the stroma (middle right). Excited chlorophyll (Chl) in its triplet state at the light-harvesting complex (LHC) can generate $^1O_2$ when the electron transport chain is over-reduced. ROS produced in peroxisomes during fatty acid oxidation, photorespiration or other reactions are decomposed by SOD, catalase (CAT) and ascorbate peroxidase (APX) (top right). SOD and other components of the ascorbate–glutathione cycle are also present in mitochondria. In addition, AOX prevents oxidative damage in mitochondria (bottom right). In principle, the cytosol contains the same set of enzymes found in the stroma (bottom left). NADPH oxidases [respiratory burst oxidase homologs (RBOHs)] are the major producers of ROS-associated signals required in a wide range of biological activities. The enzymatic components responsible for ROS detoxification in the apoplast and cell wall (CW) are only partially known, and the ROS-scavenging pathways at the vacuole and nucleus are unknown. Membrane-bound enzymes are depicted in white, GPX pathways are indicated by dashed lines and PrxR pathways are indicated by dotted lines in the stroma and cytosol. Although the pathways in the different compartments are mostly separated from each other, H_2O_2 can easily diffuse through membranes and antioxidants such as glutathione and ascorbic acid can be transported between the different compartments. DHA, dehydroascrobate; DHAR, DHA reductase; FD, ferredoxin; FNR, ferredoxin NADPH reductase; GLR, glutaredoxin; GR, glutathione reductase; GOX, glycolate oxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; IM, inner membrane; IMS, IM space; MDA, monodehydroascorbate;MDAR, MDA reductase; PGP, phosphoglycolate phosphatase; PM, plasma membrane; PSI, photosystem I; PSII, photosystem II; RuBP, ribulose-1,5-bisphosphate; Rubisco, RuBP carboxylase oxygenase; Trx, thioredoxin; tyl, thylakoid. (from Miller et al.,2009)

1.3.1.3.1.2 Superoxide dismutase (SOD1.15.1.1)

Superoxide dismutase (SOD), which dismutates $O_2^{\cdot -}$ into H_2O_2, is present in all aerobic organisms (Scandalios,1993). The enzyme SOD is classified into three groups: The Fe-SOD present mainly in chloroplasts; Mn-SOD in peroxisomes and mitochondria, Cu/Zn-SOD in the cytosol and chloroplasts.

The data of SOD enzyme activities have shown an increase under various environmental stresses such as drought and salinity. The SOD activity increase is correlated to plant capacity to face environmental stress. For instance, in case of salinity the halophytes show a greater SOD enzyme activity if compared to glycophytes (Jithesh *et al.*, 2006; Uzilday *et al.*, 2014)

1.3.1.3.1.3 Catalase (CAT,1.11.1.6)

The enzyme CAT is involved in decomposition of H_2O_2 into water and oxygen (Willekens *et al.*, 1997). The enzyme CAT has a very high turnover rate. As SOD also CAT has different isoforms, but the main one is sited in peroxisomes. The enzyme CAT is able to scavenge H_2O_2 produced by various reactions: photorespiratory oxidation, β-oxidation of fatty acids, and other enzyme systems such as XOD coupled to SOD (Corpas *et al.*, 2008; del Rio *et al.*, 1977). Some studies have shown that CAT-deficients plants had an increased susceptibility to salt, ozone and paraquat (Willekens *et al.*, 1997).

1.3.1.3. 1.4 Guaiacol Peroxidase (GPX,1.11.1.7)

Guaiacol peroxidase (GPX, EC 1.11.1.7), belongs to a family of enzymes present in fungi, plants and vertebrates. These proteins contain an heme group and oxidize several substrates such as guaiacol and pyragallol in the presence of H_2O_2 (Penel *et al.*, 1992; Vianello *et al.*, 2007). Different isoenzymes of GPX were found in vacuoles, cell wall and cytosol. GPX is involved in different biosynthetic processes including lignification of cell wall, degradation of IAA and protection against abiotic and biotic stress.

1.3.1.3. 1.5 Ascorbate-Glutathione Cycle

The ascorbate-glutathione (AsA–GSH) cycle plays an important role in the scavenging system for ROS. This pathway includes four enzymes such as ascorbate

peroxidase (APX), monodehydroascorbate reductase (MDsAR), deydroascorbate reductase (DAsAR) and glutathione reductase (GR). APX reduces H_2O_2 to H_2O, prevents the accumulation of toxic level of H_2O_2, while GR reduces oxidized (GSSG) to GSH using NAD(P)H as an electron donor. The Figure 6 schematizes the ascorbate-glutathione cycle.

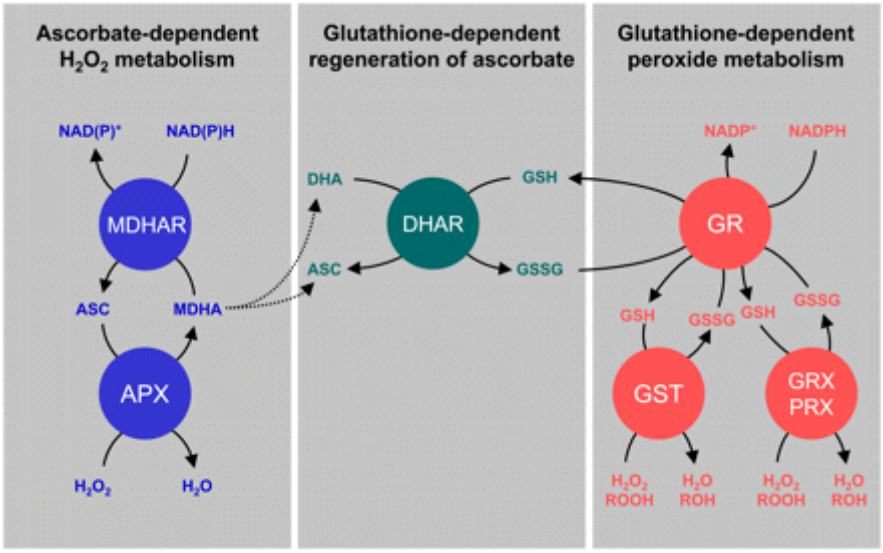

Figure 6: Ascorbate-Glutathione cycle (from Foyer and Noctor 2011)

1.3.1.3.2 Non-enzymatic control of ROS level

Ascorbate and glutathione can reduce the superoxide produced by Mehler reaction (Noctor and Foyer, 1998). Plants have evolved non-enzymatic antioxidants that together with the enzymatic pathways play an important role in ROS scavenging under normal and stress conditions for example drought. The major non-enzymatic antioxidants are mentioned below.

1.3.1.3. 2.1 Glycine-Betaine

Glycine-betaine are compatible solutes accumulated in response to various environmental stresses such as drought, salt, or cold (Mahajan and Tuteja, 2005). They are associated with osmotic balance and can have a role in ROS homeostasis. Glycine-betaine stabilize and enhance both structure and function of oxygen-evolving complex of PSII against dissociation of the regulatory extrinsic proteins (the 18 kD, 23 kD and

33 kD proteins (Papageorgiou and Murata, 1995). Exogenous application of Glycine-betaine to Arabidopsis roots, that does not produce these compounds constitutively, has been shown to alleviate OH-generated K^+ leakage (Cuin and Shabala, 2007). Moreoveor, Booker e*t al.*(2009) have shown that overexpression of glycine-betaine synthesing enzyme Betaine aldehyde dehydrogenase (BADH) in Arabidopsis and rice(non-accumulators of glycine-betaine) results in an enhanced salinity and ozone stress tolerance in transgenic plants (Booker *et al.*, 2009)

1.3.1.3. 2.2 Proline

Proline is known to accumulate in plants in response to environmental stress and represents a solute involved in plants protection from water deficency. Chen and Dickman (2005) demonstrated that the amino acid proline is a potent scavenger of ROS (Chen and Dickman, 2005). Moreover, this amino acid is capable to mitigate the impacts of drought, salt and temperature stress in plants. For example, proline accumulation can influence stress tolerance in multiple ways (Fig 7).

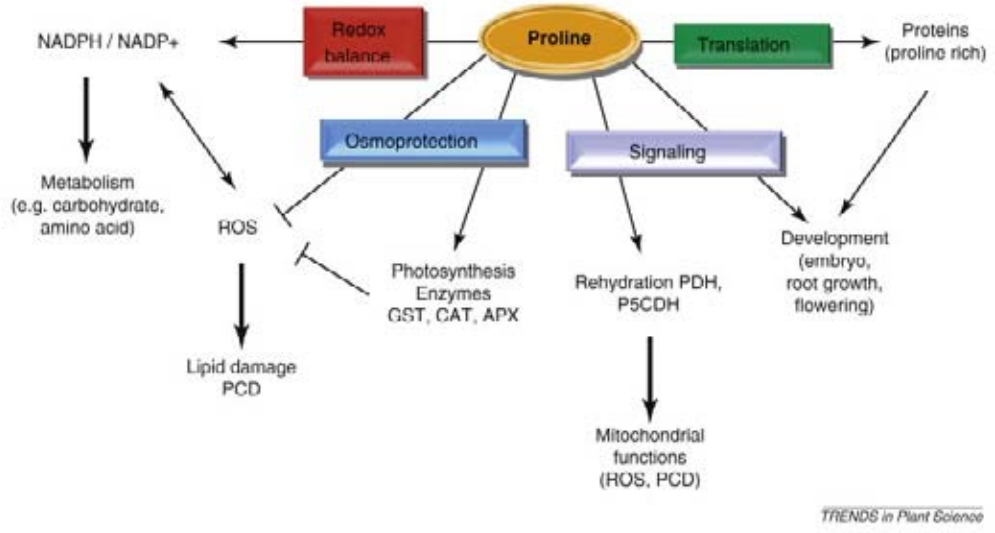

Figure 7: Multiple functions of proline in plants. Proline is used for protein synthesis, has protective functions as an osmolyte, contributes to the maintenance of the redox balance, can regulate development and is a component of metabolic signaling networks controlling mitochondrial functions, stress relief and development. Abbreviations: APX, ascorbate peroxidase; CAT, catalase; PCD, programmed cell death (from Szabados and Savouré, 2010)

1.3.1.3. 2.3 Polyamines and other secondary metabolities

Polyamines (PA) are polycationic compounds present in most living organisms (Alcázar *et al.*, 2010). These compounds seem to be involved in plant growth and development, PCD, signaling, gene expression and adaptation to environmental stresses (Kuznetsov and Shevyakova, 2007; Takahashi and Kakehi, 2010). Recently it was shown an important role for 1,3-diaminopropane (DAP) in A. thaliana drought stress response (Jammes *et al.*, 2014). The 1,3-diaminopropane in plants is co-produced with H_2O_2 from metabolism of the classical polyamines. Acetylated 1,3-diaminopropane seems to have a role in adaptation to drought avoiding the complete stomatal closure to maintain CO_2 diffusion to photosynthetic tissues. Indeed, during stomata closing there is an increase in water use efficiency for the plant and the CO_2 is cut off. This diminution in CO_2 may conduce to stomatal (re)opening despite high ABA and the increased liability of dehydration, and even death (Jammes et al.,2014). The model proposed by Jammes et al. (2014) shown that during slow-soil-water availability, acetylation of DAP by acetyltransferases, identified earlier as NATA1 is involved in regulation of membrane transport mechanisms involved in readjusting CO_2-H_2O homeostasis. While an intense drought leads to suppression of NATA1 expression (Jammes *et al.*, 2014).
Carotenoids are natural pigments, which are synthesized by plants, with antioxidant property, indeed they are involved in quenching of $^1O_2$ and peroxy-radicals (Neubauer and Yamamoto, 1994). Other secondary metabolites such as sorbitol, mannitol, myo-inositol, ononitol or pinotol are accumulated during drought and salinity stresses (Williamson *et al.*,2002) acting as antioxidants.

1.3.2 Calcium signaling in response to osmotic stress

Calcium (Ca^{2+}) is an important second messenger in regulation of different cellular processes and Ca^{2+}dependent signaling is widely conserved among eukaryotes. (Lecourieux *et al.*, 2006; Xiong *et al.*, 2014).

In plant cells, $[Ca^{2+}]_{cyt}$ is maintained low around 100nM respect to the vacuole (200 nM) and apoplast (300 nM) and it was proposed that this mechanism may provide the possibility to quickly import calcium inside the cell when required for signalling (Lecourieux *et al.*, 2006). Changes in plant $[Ca^{2+}]_{cyt}$ are observed in response to salinity and drought (Parre *et al.*, 2007; Ranf *et al.*, 2008). The signalling pathway in which is involved Ca^{2+} is complex because it processes multiple signals simultaneously. Increase in $[Ca^{2+}]_{cyt}$ are sensed by Ca^{2+}-sensor proteins and it transforms them in downstream pathways by binding or activating different targets.

Among the effector proteins there are calmoduline (CaM), CaM-binding proteins, Ca^{2+}-dependent protein kinases(CDPK) (Wheeler and Brownlee, 2008). The signals from CaM can induce gene expression of specific transcription factors (Tuteja and Mahajan, 2007). CDPKs were found in osmotically stressed plants, in fact a CDPK was reported to be expressed earlier and for longer in a drought tolerant species respect to the sensitive ones. In plant several isoforms of CDPKs exist and this can be explained with the need of fine tuning responses to diverse abiotic stresses.

Calcium permeable channels can be considered as the upstream elements in the Ca^{2+}-dependent signalling pathways, thus their activation is quite important. These channels are placed in plasma membrane, tonoplast, as well as in the ER (Kudla *et al.*, 2010), and their activity is coupled with Ca^{2+}-ATPase whose main function is to maintain calcium homeostasis by pumping out calcium (White, 2000). On plasma membrane also Ca^{2+} voltage dependent channels are present and their activation depend on depolarization of membrane such as depolarization-activated Ca^{2+}channel (DACC)and the hyperpolarization-activated Ca^{2+}channel (HACC) (Tuteja and Mahajan, 2007). Cytosolic calcium increase can be due also to Ca^{2+} mobilization from organelles, event mediated by second messengers, IP3or cADPR ((Hetherington and Brownlee, 2004), that acts as ligand for calcium gated channels in vacuolar or ER membranes or by Ca^{2+}-antiporters (Lecourieux *et al.*, 2006).

It has also been reported that the rise in $[Ca^{2+}]_{cyt}$ in plant could be provided by non-selective cation channels (NSCCs) that allow the cation passage across membranes with limited discrimination between cations (Demidchik and Maathuis, 2007). These NSCCs play also a crucial role in salinity stress (Demidchik and Maathuis, 2007). Indeed, Ca^{2+} blocks some NSCC and consequently reduces Na^+ influx into intact tissue to maintain a correct K^+/Na^+ ratio (Demidchik and Maathuis, 2007).

A recent study has demonstrated that Ca^{2+}-dependent wave signaling may play an important role in the process of systemic signaling in response to salt stress (Choi *et al.*, 2014). It was found that local salt stress at the root tip level leads to the spreading of a Ca^{2+} wave that propagates preferentially through cortical and endodermal cells to distal shoot tissues, triggering systemic molecular responses in the shoot. In particular, local treatment with $LaCl_3$, a putative plasma membrane channel blocker, inhibits the transmission of the Ca^{2+} waves and in parallel also inhibits the systemic molecular responses. Up to now, the Cyclic Nucleotide-Gated Channels(CNGC) and the Glu Receptors (Dodd *et al.*, 2010) involved in Ca^{2+}flux (Vincill *et al.*, 2012) are identified, but none have been shown to function in NaCl-induced $[Ca^{2+}]_{cyt}$ elevation. Recently a role was proposed for plant anexin in Ca^{2+}flux under salinity (Laohavisit *et al.*, 2013). The anexin1 (AtANN1) seems to have an role in the generation of root and root epidermal NaCl-induced $[Ca^{2+}]_{cyt}$ elevation.

1.3.2.1 Polyphosphoinositides (PPIs)

Polyphosphoinositides (PPIs) are present in all eukaryotes (Michell, 1975). The Phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P2] is the best known PPI and it is the substrate for signal-activated phospholipase C (PLC), with the latter being an enzyme that hydrolyzes inositol-1,4,5-trisphosphate (InsP3) and diacylglycerol (DAG). InsP3 releases Ca^{2+} from an intracellular store by activating a ligand-gated Ca^{2+}-channel (Munnik and Vermeer, 2010) which triggers several physiological changes in response to osmotic stress, including stomata closure (Bartels and Sunkar, 2005). Furthermore, in plant the first phospholipid based signalling pathway in response to osmotic stress is the PLC pathway, which is activated within minutes after both salt and water stresses (Munnik and Vermeer, 2010). Osmotically-triggered PtdIns(3,5)P2 responses have been reported for *Chlamydomonas*, tobacco pollen tubes, epidermal

strips of pea leaves and suspension-cultured cells of tomato and alfalfa (Munnik and Vermeer, 2010; Zonia and Munnik, 2004). In mammalian cells, during osmotic stress, the PtdIns (4,5) P2 regulates many ion channels and transporters (for example NHE1, VRAC, ENaC) (Hilgemann *et al.*, 2001; Suh and Hille, 2005; Voets and Nilius, 2007). All phosphatidylinositols phosphorylated at position 3 are synthesized by the phosphatidylinositol 3 kinase (PI3K), capable of phosphorylating inositol in position D3 (Toker, 1998). Several studies suggest that the activity of this enzyme is related with the Regulatory Volume Decrease Channel (RVDC, topic discussed more in detail in the next section). Current knowledges indicate that the PI3K plays a role as a sensor of cellular swelling, as the phosphorylation of PI in position 3 of the ring, by means of PI3K, strongly depends on the curvature of the membrane; therefore it would appear that the activity of this enzyme can be regulated by the mechanical deformation of the membrane itself due to changes in cell volume (Hübner *et al.*, 1998).

1.3.2.3 Salt Overly Sensitive (SOS)

Exposure to salinity activates the Salt Overly Sensitive (SOS) pathway. The SOS pathway is involved in removal of Na^+ from the cytosol by activation of Na^+/K^+ exchangers (antiporters), which are membrane proteins localized in the vacuolar or plasmamembrane. The Ca^{2+} spike after salt stress induces the SOS pathways. SOS3 encodes amyristoylated calcium-binding protein that appears to have the function of calcium sensor in the perception of an increase of cytosolic Ca^{2+}. The complex SOS3-Ca^{2+} is able to interact with the protein kinase SOS2 which phosphorylates SOS3 proteins. SOS3–SOS2 interactions recruit SOS2 to the plasma membrane, leading to activation of the downstream target SOS1, an Na^+/H^+ antiporter allowing extrusion of excessive Na^+ from the cytosol (Ji *et al.*, 2013).

1.3.4 Protein kinases

1.3.4.1 MAPKs pathway

Mitogen-activated protein kinase (MAPKs) appears to be a key mechanism for intracellular signal transduction in both eukaryotic and prokaryotic. In general, the cascades of MAP kinase enzymes are characterized by the presence of three functionally related protein kinases: (i) the activation of MAP kinase requires phosphorylation of tyrosine and threonine residues, preserved in the so-called "TEY (Thr,Glu,Tyr) activation loop" by the MAPK kinases (MAPKK); (ii) in turn, the MAPKK are activated through phosphorylation of threonine residues of conserved threonine and or serine by a MAPKK kinase (MAPKKK) (Bartels and Sunkar, 2005); (iii) and finally the activated form of MAPK then migrates from the cytoplasm to the nucleus in order to directly activate transcription factors (TF). In vertebrates 14 MAP kinase genes have been identified that define 7 distinct MAP kinase pathways, involved in different abiotic and biotic stresses (Pearson *et al.*, 2001). For example, in vertebrates, TF involved in osmotic stress response is the NFAT5 that activates the transcription of genes, which are indirectly involved in cell volume regulation, such as the sodium chloride-betainecotransporter (SLC6A12)the sodium/myo-inositol cotransporter (SLC5A3) and the taurine transporter (SLC6A6) (Lee *et al.*, 2011). As in vertebrates, in plants there are several MAP kinase genes, that define distinct MAP kinase pathways vital in the signaling of different abiotic and biotic stresses. For example, in *Arabidopsis thaliana* there are MPK3, MPK4 and MPK6 kinases involved in response to shock osmotic stress. Indeed, Kim and coworkers (Kim *et al.*, 2011) have shown a role for MPK4, and observed that mpk4 loss-of-function mutants and MpK4-overexpressing transgenic plants (MpK4 OXs) produce aberrant responses following osmotic stress. In-gel kinase assays for MPK3 phosphorylation in mpk4 mutants and MpK4 OXs demonstrated that MpK4 plays an important role in osmotic-stress responses via regulation of MPK3 activity; in fact, after osmotic stress in Mpk4 mutant, there is a decline in phosphorylation rate meanwhile in MpK4 OXs the level of phosphorylation remains higher, even when compared with wild type. In addition, it was also found that these components also cross-talked with ABA-dependent stress-responses genes, in particular NCED3, a gene that encodes a key regulator of the ABA signaling pathway. NCED3 gene expression is reduced in mpk4 mutants and increased

in MpK4 OXs compared with WT plants. All together, these results strongly suggest that MpK4 might mediate osmotic-stress response and promote NCED3 expression(Kim *et al.*, 2011).

1.3.5 Plant ion channels

The ion channels activity is generally associated to the earliest observed events following the perception of a stimulus. The ions (eg. K^+,Na^+, etc) are transferred in the direction of their electrochemical gradient, from one compartment to another. The opening/closure of these channels depends on various stimuli, there are two main types of ion channels:gated and non-gated. Under physiological conditions, ion channels perform three basic functions: (i) are involved in osmotic homeostasis (ii) the transduction of signals, amplifying and propagating electric signals for example, or carrying secondary messengers, and (iii) control of the membrane potential. The following sections describe an overview of the different classes of ion channels present in plants, focusing mainly on plasma membrane channels, on how their structures and activities are linked to other signaling events, and finally, on their roles and implications in signaling pathways leading to PCD process.

1.3.5.1 Plant cation channels

1.3.5.1.1 Potassium channels

Potassium (K^+) represents up to 10% of the total dry weight of the plant. It is the most abundant cation in the cytosol as it is compatible with the structure of proteins, even at high concentrations (Kronzucker and Britto, 2011; Maathuis and Amtmann, 1999). The cell uses this cation in several important functions such as neutralization of the anion electric control of membrane polarization and osmoregulation. Plants have a variety of transport systems for K^+(Fig.8).

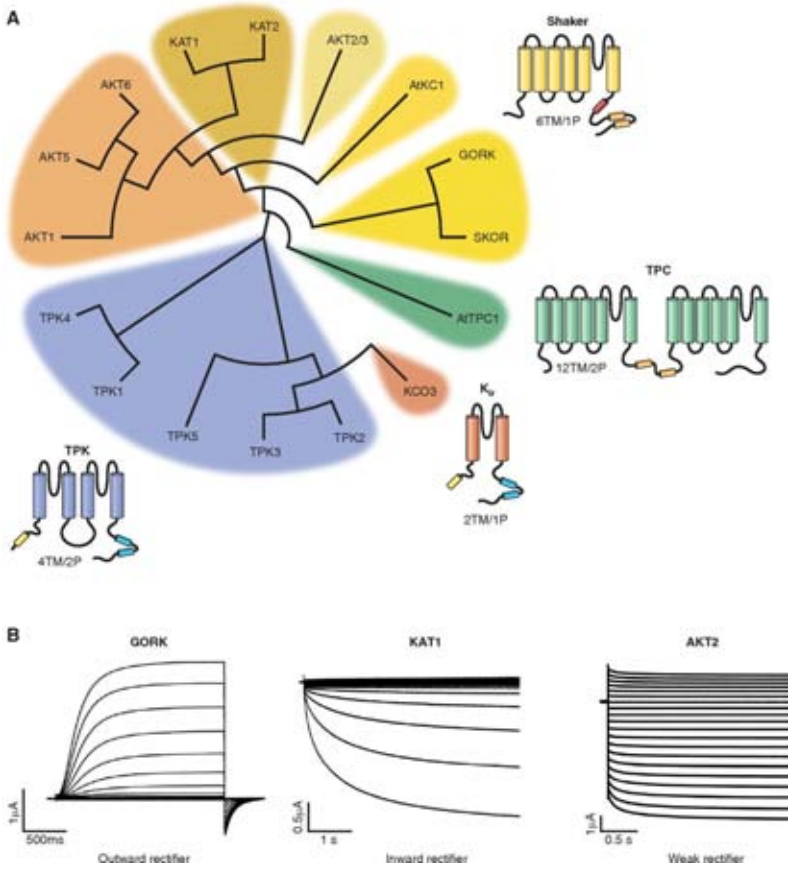

Figure 8:Phylogenetic relationship and topology of *Arabidopsis thaliana* potassium channels. A: the genome of the model plant Arabidopsis encodes for 15 K^+-selective channels, which are subdivided into three structural classes: The class of voltage-dependent *Shaker*-like potassium channels (K_v), the twin pore channels (TPKs;K_{2P}), and K_{ir}-like potassium channel (KCO3). In addition, it encodes for a cation-nonselective two-pore channel (TPC1). TM, transmembrane domain; P, pore region. B: despite their structural resemblance, plant Shaker-like K_v channels split up into outward (*left*), inward (*middle*), and

weak (*right*) rectifiers according to their gating properties. Typical voltage-clamp recording from heterologously (Xenopus oocytes) expressed Kv channels are shown (from Hedrich,2012)

In plants, as in animal cells, the activity of potassium channels is determined by the cell membrane electrical properties. Two K^+ permeable conductances have been characterized and they differ in the voltage range by which they are activated. One is activated by membrane hyperpolarization (with potential more negative than $E_K = E_i$, thermodynamic equilibrium potential of K^+) and it is mainly involved in K^+ entry (Fig 9). This incoming rectifying conductance is commonly called IRC Inward Rectifying Conductance (Fig 10). The other K^+ conductance is activated at potential levels more positive than E_K membrane and it's involved in the efflux of K^+ out of the cell. This type of conductance is called ORC for Outward Rectifying Conductance.

$$E_i = \frac{RT}{zF} \ln \frac{[i]_{int}}{[i]_{ext}}$$

Figure 9: Nernst Equation

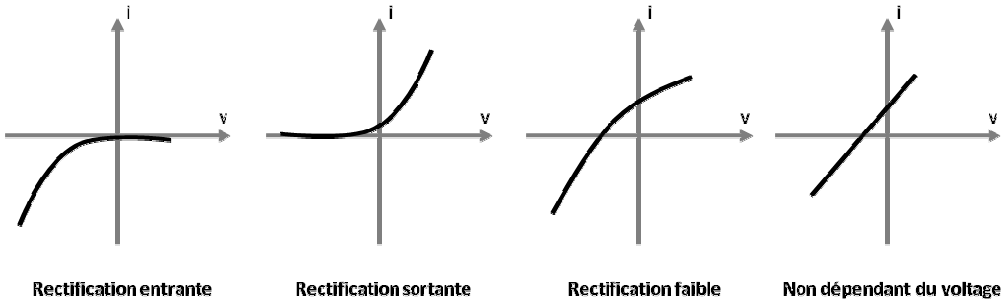

Figure 10: Relation between current and voltage (I/V). It is shown the different type of K^+channels in plants (Adapted from Véry & Sentenac, 2002)

The ORC is characterized by slow sigmoidal activation current (Fig 8). The ion channels belonging to this family are mainly selective for K^+ if compared to Na^+ and are blocked by Ba^{2+} and tetraethylammonium (TEA). In Arabidopsis, the presence of ORC was reported in guard cells, root hairs and cultured cells of different plants (Bouteau and

Tran, 2012; Forestier *et al.*, 1998; Roelfsema and Hedrich, 2010). The K^+ channels are multimeric proteins. Their transmembrane subunits, named α subunits are characterized by the presence of one or two pore domains P. Three families of subunits α forming selective channels K^+ have been identified in plants areas: Shaker, TPK and Kir-like (Véry and Sentenac, 2003). They all have counterparts in animal cells.

3.5.1.2 Shaker-like potassium channels

Shaker-like potassium channels, inhibited by TEA, are divided in three groups depending on their voltage sensitivity (Véry and Sentenac, 2002). The first class is called Potassium Inward-Rectifying channels (KIR) including *AKT1, KAT1, KAT2,* and *SPIK*. They are responsible of K^+ uptake after membrane hyperpolarization. In the second class there are the potassium channels that depending in K^+ electrochemical gradients mediate both uptake and release.*AKT2* belongs to this family. The third is the Potassium Outward-Rectifying channels (KOR) including *GORK* and *SKOR* that mediate K^+ release upon membrane depolarization. The Shaker family contains nine genes and are composed of four subunits arranged around a central pore (Zimmermann and Sentenac, 1999).Seven of the nine Shaker Arabidopsis proteins were characterized so far and it was found to be highly selective for K^+. The available information suggests that these channels are the main transport systems involved in the nutrition K^+ and / or the regulation of the K^+ equilibrium. Among the Shaker ion channels GORK(outward-rectifying potassium-selective channel) is expressed in guard cells and root hairs, while SKOR is localized in the vasculature, namely, the xylem parenchyma cells, where it is involved in solute loading in the xylem network (reviewed by, (Hedrich, 2012). Fig. 11 shows a schematic representation of the expression patterns of Arabidopsis K^+ - selective channels.

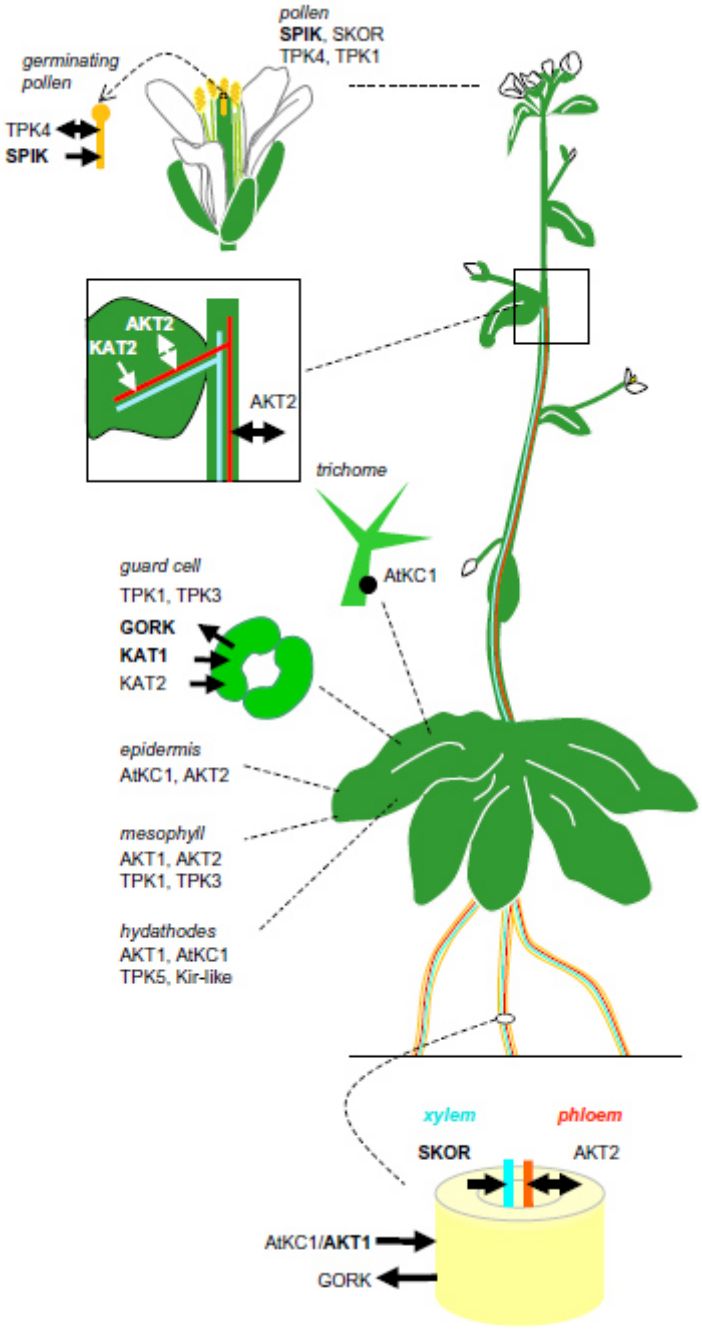

Figure 11: Expression patterns of Arabidopsis K+-selective channels (from Lebaudy et al.,2007).

1.3.5.1.3 Twin-Pore K^+/KCO

In mammalian cells, the KCNK are characterized by a hydrophobic core composed by four transmembrane segments and two P domains (Fig 12). The characteristic of this class of ion channel is that they have a weak voltage activation (Patel and Honore, 2001). In Arabidopsis, five channels (TPK1, TPK2 ,TPK4, and TPK5 TPK6) have structural homologies to mammalian ion channels (Voelker *et al.*, 2006; Zimmermann and Sentenac, 1999). These channels are localized in the tonoplast (Czempinski *et al.*, 2002; Schönknecht *et al.*, 2002; Voelker *et al.*, 2006), except AtTPK4, which is localized at the plasma membrane and it's specific for the pollen tube (Becker *et al.*, 2004).

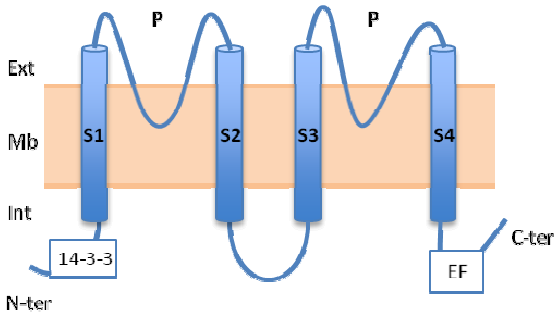

Figure 12:Structural conformation of TPK ion channels in Arabidopsis (from Lebaudy et al., 2007)

1.3.5.1.4 Kir-like

In mammalian cells, this family is characterized by IRK conductance and has an hydrophobic core formed of two segments and a trasmembrane domain (fig 13) .These channels don't show voltage-dependence, the inward rectification is due to a blockage of outward current by Mg^{2+} and polyamines (Ficker *et al.*, 1994; Matsuda *et al.*, 1987). In *Arabidopsis thaliana*, a channel located at the tonoplast was found (Voelker *et al.*, 2006) to share similarity with the two P domain channels (KCO family) and was therefore named KCO3 (Czempinski *et al.*, 1999).

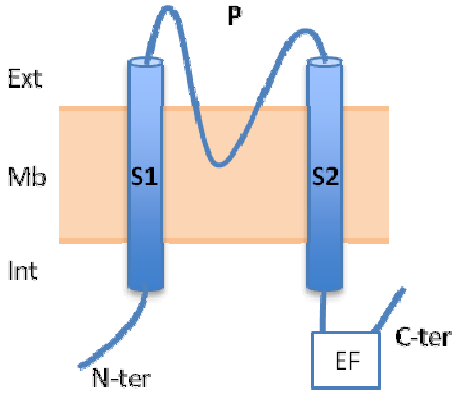

Figure 13: Structural conformation of *Kir-like* ion channels in Arabidopsis (Adapted from Lebaudy et al., 2007)

1.3.5.1.5 Non selective cation channels (NSCC)

Non-selective cation channels are macromolecular pores at plasma membrane that allow the ions Na^+, K^+, Ca^{2+} fluxes.

Under saline conditions Na^+ enters in the cells through non-selective cation channels (NSCCs,) (Demidchik and Tester, 2002), depolarizing the plasma membrane (Chen *et al.*, 2007; Pandolfi *et al.*, 2010; Shabala and Cuin, 2008; Wegner *et al.*, 2011).

Even though no definitive molecular candidates have clearly emerged for NSCCs, it seems that various classes of NSCCs could be responsible for influx of Na^+ under salt stress, especially a depolarization activated class of NSCCs (Demidchik and Maathuis, 2007). There are some candidate genes belonging to family of glutamate receptors (GluR) (Lacombe *et al.*, 2001) and the family of CNGC (= Cyclic Nucleotide Gated Channel) (Mäser *et al.*, 2001). The NSCC may be activated by many factors such as the voltage (hyperpolarization or depolarization), calcium, mechanical variations, cyclic nucleotides or glutamate (Demidchik *et al.*, 2002). Most studies have focused on the NSCC of roots. They are responsible for the influx of Na^+ at the root level (Demidchik and Maathuis, 2007; Demidchik and Tester, 2002). NSCCs can therefore be subdivided according to their voltage dependence into voltage-insensitive, depolarization-activated o rhyperpolarization-activated NSCCs (reviewed by(Demidchik and Maathuis, 2007)):

- Depolarization-activated NSCCs (DA-NSCCs)
- Voltage-independent NSCCs (VI-NSCCs)
- Hyperpolarization-activated NSCCs (HA-NSCCs)

The DA-NSCCs were found in *Arabidopsis* cultured cells(Cerana and Colombo, 1992), *Arabidopsis* leaf mesophyll (Shabala *et al.*, 2006), Arabidopsis root epidermis (Shabala *et al.*, 2006), *Arabidopsis* guard cells (Pei *et al.*, 1998), *Hordeum vulgare* root xylem (Wegner and De Boer, 1997), *Thlaspi* spp. Mesophyll cells (Piñeros and Kochian, 2003), *Arabidopsis* pollen tubes (Becker *et al.*, 2004), *Phaseolus vulgaris* seed coats (Zhang *et al.*, 2002) and *Phaseolus vulgaris* cotyledon epidermal cells (ZHANG *et al.*, 2004). The voltage dependence of DA-NSCCs is similar to outward rectifying K^+-selective channels (ORC). The DA-NSCCs can be blocked by extracellular Ca^{2+}, TEA, nifedipine, diltiazem and verapamil (reviewed by (Demidchik *et al.*, 2002; Shabala *et al.*, 2006).

The voltage-independent NSCCs (VI-NSCCs) are insensitive to plasma membrane voltage, this suggests that they equally conduct outward and inward currents. They can be divided into two groups on the basis of blockage by cations such as Ca^{2+}, Ba^{2+},Mg^{2+} and Zn^{2+}. This class of ion channels was found in Aster tripolium guard cells (Véry *et al.*, 1998), *Arabidopsis* mesophyll cells (Shabala *et al.*, 2006), Arabidopsis cells (Amtmann *et al.*, 1997), *Arabidopsis* pollen tubes (Becker *et al.*, 2004), expanding *Pisum sativum* leaf epidermis (Elzenga and Van Volkenburgh, 1994) and motor cells of *Samanea saman* (Yu *et al.*, 2001). DA-NSCCs and VI-NSCCs are the primary candidates for Na^{+} entry.

The Hyperpolarization-activated NSCCs (HA-NSCCs) conduct mainly Ca^{2+} and were described as hyperpolarization-activated Ca^{2+} channels (HACaCs) (Demidchik and Maathuis, 2007). This class of NSCC is involved in Ca^{2+} influx important for plant elongation, growth of root hairs and cells in the elongation zone (Demidchik and Maathuis, 2007). Gelli *et al*. (Gelli *et al.*, 1997) have shown that some type of HA-NSCCs are sensitive to elicitors, this suggest that these channels may be involved in Ca^{2+}signalling, which is part of the early response to pathogen attack (Gelli *et al.*, 1997).

1.3.5.2 Anion channels

Anion currents are divided into S-type, R-type, and IRAC. Guard cells were known to have both S- and R-type anion channels (Fig 13,14)(reviewed by (Hedrich, 2012)

1.3.5.2.1 Slac

Slac1 (slow-associated Anion Channel 1) (Fig 14), was identified from *Arabidopsis* mutant, which encodes a protein from the plasma membrane in guard cells (Negi *et al.*, 2008; Vahisalu *et al.*, 2008).

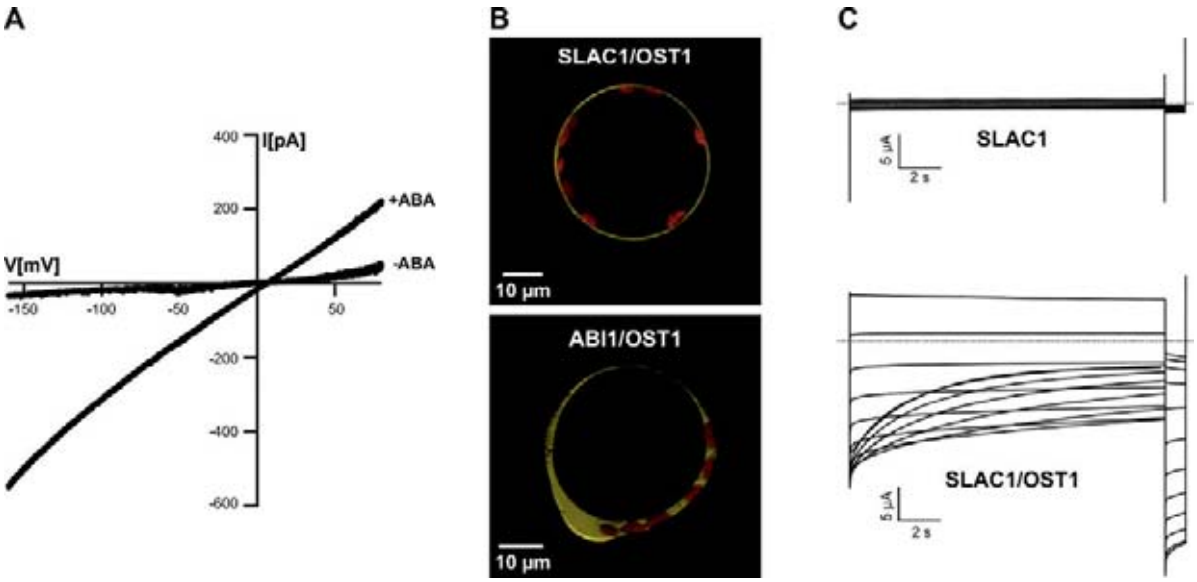

Figure 14: Anion channel SLAC1 controlled by protein kinase-phosphatase. A: S-type anion channels in the plasma membrane of Vicia faba guard cells are activated by stress hormone ABA. B split YFP assays: top panel, SLAC1, a guard cell plasma membrane S-type anion channel, is interacting with the SnRK2 protein kinase OST1 at the plasma membrane. Bottom panel: the cytosolic enzymes OST1 (protein kinase) and ABI1 (2C protein phosphatase) physically interact. C: two electrode voltage-clamp experiments indicate that SLAC1 activation in Xenopus laevis oocytes requires OST1. (from Hedrich,2012)

The mutated alleles slac1 show insensitivity to CO2, ABA and O_3-induced stomatal closure (Negi *et al.*, 2008; Vahisalu *et al.*, 2008). Analyses in patch-clamp the two

mutants slac1 alleles, showed that slow anionic currents were considerably reduced, while current rapid anion channels (R-type) and permeable channels Ca^{2+} of the plasma membrane were intact in guard cells.

SLAC1 belongs to gene family composed by five members (SLAH1, 2, 3 and 4). SLAC1 gene encodes a membrane protein that has ten transmembrane domains with large N and C terminal domains (Fig 15). The SLAC proteins have low homology in their transmembrane domains with a malate transporter of fungi and bacteria of the TDT family (for Tellurite-resistance/Dicarboxylate Transporter) (Camarasa *et al.*, 2001). This protein is controlled by the torque kinase OST1 (Open stomata 1) and PP2C in stomatal response, but also by a kinase CDPK (Geiger *et al.*, 2010). These channels are the main actors of drought-induced membrane depolarization and it was shown that in root hairs they are activated during desiccation (Dauphin *et al.*, 2001).

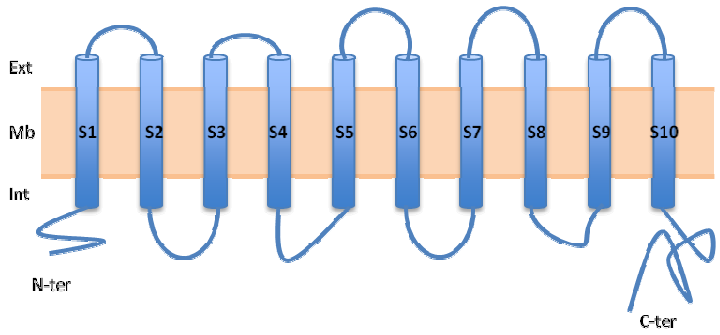

Figure 15: SLAC1 structure (from Chen *et al.*,2010)

1.3.5.2.2 IRACs channels

The IRACs channels were found in various species of plants and algae, as in pea (*Pisum sativum*), bean (*Phaseolus vulgaris*), Medicago sativa (Bouteau *et al.*, 1999), Arabidopsis thaliana (Bouizgarne *et al.*, 2006), barley (*Hordeum vulgare*), phytoplankton (*Coccolithus pelagicus*), seaweed *Valoniautricularis* (Tavares *et al.*, 2011*)* and root hairs of Medicago sativa (Bouteau *et al.*, 1999). These channels share a voltage-dependent inward rectification. In maize (*Zea mays*) and barley, the inward rectification is amplified by the increasing cytosolic Ca^{2+}, but the open mechanism is different (Gilliham and Tester, 2005; Köhler and Raschke, 2000). In maize, the IRAC channel is voltage-dependent (open probability increases at negative voltages). These

channels are responsible for maintaining the homeostasis of the cell anionic e.g. in the salt stress tolerance during the repolarization of the membrane potential and in regulating the osmotic stress caused by drought or excess of water. It also helps preventing strong depolarizations / hyperpolarizations of the plasma membrane. To date no molecular data are available on this type of channels.

1.3.5.2.3 R-type anion channels

The R-type anion channels (Fig 16) are characterized by rapidity of anion efflux. These channels were found in guard cells of Vicia faba (Raschke *et al.*, 2003) as well as the rapid anion channel GCAC1 (Guard Cell Anion Channel 1) (Geiger *et al.*, 2009). Under normal conditions R-type channels are closed, while a depolarization induced channels opening.

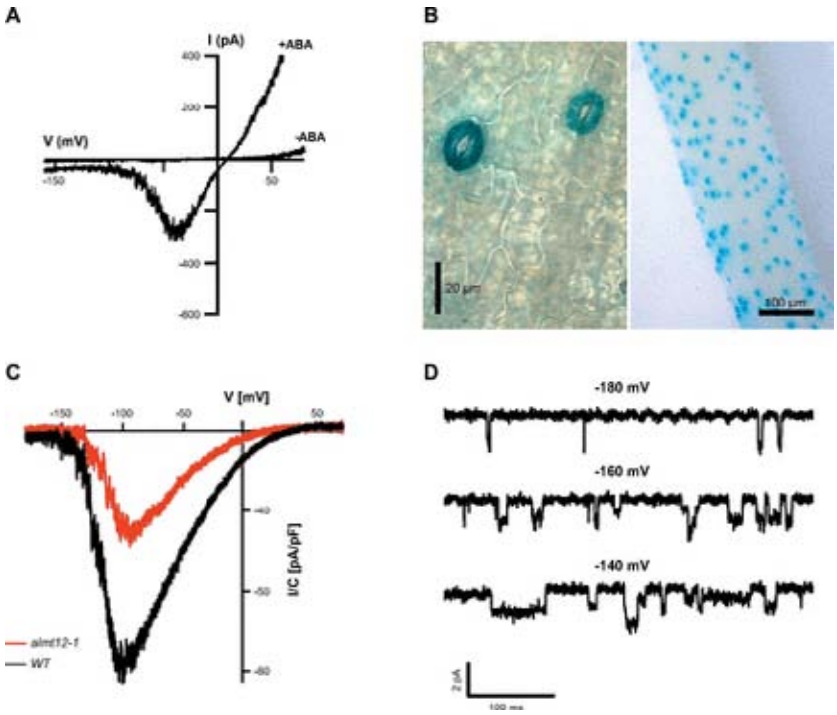

Figure 16:R-type anion channels gate like cation channels in nerve cells. A: R-type anion channels in the plasma membrane of Vicia faba guard cells are activated by ABA and gated open by depolarization (Hedrich,2012)

1.3.5.3 Mechanosensitive ion channels

In bacterial and mammalian cells, the mechano-sensitive ion channels (MS) are involved in the perception of changes in pressure, touch and sound (Hamill and Martinac, 2001; Vogel and Sheetz, 2006; Zhou *et al.*, 2005). Although plants respond to a wide variety of mechanical stimuli and many mechano-sensitive ion channel activities have been characterized in plant membranes by the patch-clamp method, the molecular mechanisms of mechanical perception remain poorly understood (Haswell, 2007). In Arabidopsis, 10 proteins MSCs (for MechanoSensitive Channel of Small conductance) have been found, called MSL1 10 (Haswell, 2007; Kloda and Martinac, 2002; Pivetti *et al.*, 2003). The structure predictions show that MSL have six transmembrane domains and parts of N-and C-b are cytosolic (Fig 17). MSL3 and MSL2 are involved in controlling the morphology of the organelles (Haswell and Meyerowitz, 2006; Nakayama *et al.*, 2007).Five proteins are found in the plasma membrane of root cells MSL4, 5, 6, 9 and 10 (Haswell *et al.*, 2008). The use of mutant lines msl9 and msl10 showed mechanical dependent channel activity with greater selectivity than the ions Ca^{2+}, Cl^- in the roots of *A. thaliana* (Haswell *et al.*, 2008).

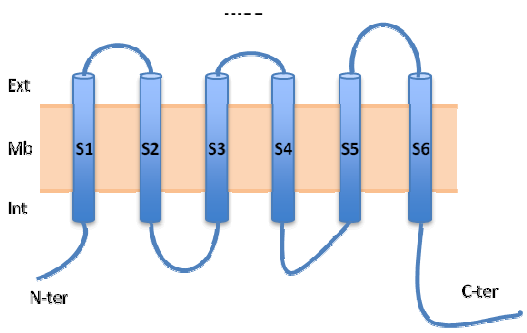

Figure 17:Mechanosensitive ion channels structure (from Lebaudy et al., 2007).

1.4 Osmotic stress, cell volume regulation and cell death

Osmotic stress induces water flux across the plasma membrane, resulting in altered cellular volume and ionic strength, and severely affects cell functions.

In general, the cell hasn't a "preferred" volume to adapt to osmotic (hyper and hypo) changes. Animal cells have evolved the capacity to change their volume, which is an important factor for cells adaptation to environment challenges. Indeed, the control of volume and osmolarity (also of turgor in plants) lies at the core of cellular homeostatic networks. These volume changes are mainly driven by K^+, Cl^- and other solutes (e.g. betaine) fluxing across the plasma membrane.In fact, a correct balance between RVI (Regulation volume increase) and RVD (Regulation volume decrease) should lead to mitigation of osmotic stress, while an excessive RVD can lead to cell death. This is an important point in cell biology that needs more studies, because it is often not very easy to understand the "switch" that lead from correct balance RVI/RVD to RVD. Therefore in this thesis we will focus on the role of ion channels in this "switch". The regulation of ion channels in apoptosis, a type of programmed cell death (PCD), was widely studied in mammalian cells (Yu and Choi, 2000), as a chronic event alters volume regulation and/or ion transporter expression, resulting in pathological developments associated with cancer (Pedersen *et al.*, 2013). The key mechanism that links ion channels, cell volume and PCD is the Apoptosis Volume Decrease (AVD) and involve K^+ and/or Cl^- fluxes (Fig 18).

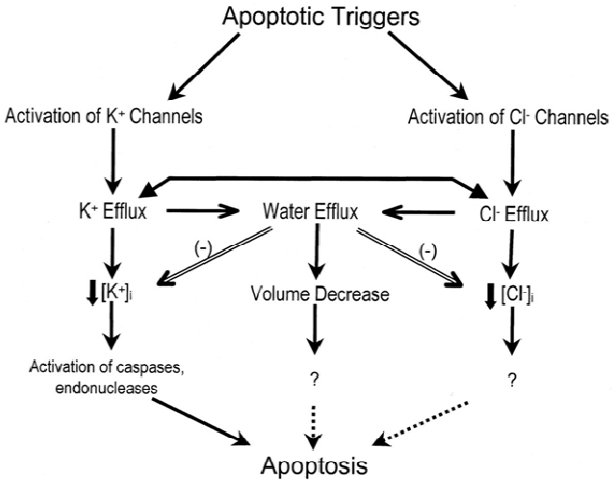

Figure 18:Mechanism of Apoptosis volume decrease involved in apoptosis (from Yu & Choi,2000).

Particularly, the diminution of intracellular K^+ leads to the caspases activation, since the intracellular K^+ promotes the catalytic activity of caspases and nucleases (Kroemer *et al.*, 1998; Perregaux and Gabel, 1994).

In animal cells, it was shown that the volume regulation could protect cells from apoptosis (Maeno *et al.*, 2000; Maeno *et al.*, 2006; Reinehr *et al.*, 2007). Numata et al (Numata *et al.*, 2007) suggested that the transition receptor channel has a role in cell volume regulation because the calcium increase, which leads to the activation of K^+ and Cl^- calcium. In mammalian cells, the $I_{Cl,sweel}$ (a commonly name used for classification of the chloride anion channels) is one of the major patways responsible for RVD (Hoffmann *et al.*, 2009; Lang *et al.*, 2007), but the molecular characteristics of channels underlying the $I_{Cl,sweel}$ (normally involved by cell swelling resulting from exposure to extracellular hypotonic solution) (Catacuzzeno *et al.*, 2014) still remain unknown.

In plant, K^+ efflux and its involvement in PCD was widely studied in response to salinity stress (as osmotic challenge) and biotic stress (i.e. in response to harpins). Indeed, in *A.thaliana* roots the K^+ efflux in response to salinity stress is necessary to lead PCD, (Demidchik *et al.*, 2010), and anion efflux coupled with potassium efflux have an important role in plant cell volume regulation. In *A.thaliana* cells the harpine N (HrpNea) produced by plant pathogenic bacteria lead to the activation of K^+ currents in the plasma membrane: K^+ outward rectifying currents (KORC) (Reboutier *et al.*, 2007). Recently, in A. thaliana was also reported a delayed KORC activation in response to ozone (O_3) (Tran *et al.*, 2013b). The exposure of *A.thaliana* suspension to O_3 induced an acute cell death (Kadono *et al.*, 2010). This cell death is characterized and involved in an early activation of anion channel (Kadono *et al.*, 2010) and a delayed activation of K^+ outward currents (Tran *et al.*, 2013a). In animals, cell shrinkage involved dysfunction of ionic homeostasis and consequently progression of cell death (e.g. caspase and nuclease activation) (Bortner and Cidlowski, 2004; Yu and Choi, 2000). It was shown that ozone induced a ion channel dependent caspase-like activities involved in early events participating to development of O_3-induced PCD (Tran *et al.*, 2013b).

During biotic stress, the anion efflux was described as an early event induced by interaction plant/pathogens in the hypersensitive response (Garcia-Brugger *et al.*, 2006) induced by harpins (Reboutier *et al.*, 2007), thaxtomine, oxalic acid (Errakhi *et al.*,

2008) or cryptogeine (Gauthier *et al.*, 2007; Wendehenne *et al.*, 2001). For example, it was found that the oxalic acid or cryptogein induced cell death depending on the activation of anion channels. It was also found that ozone induces cell shrinkage by an early activation of anion channels that leads to cell death (Kadono *et al.*, 2010).

1.5 Programmed Cell Death

The Programmed Cell Death (PCD) is an essential part of plant development and responses to abiotic and biotic stress (Williams *et al.*, 1992). This type of cell death is a genetically programmed process characterized by distinct morphological characteristics. Different types of PCD have been described in plants, which has led to a call for a detailed classification of cell death events (Van Doorn *et al.*, 2011). Typical peculiarities of PCD in plants frequently include the fragmentation of the DNA by specific nucleases (DNA laddering), condensation and shrinkage of the cytoplasm, release of cytochrome *c* from mitochondria, elevation of the cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$), generation of reactive oxygen species (ROS), and an activity increase of caspase-like enzymes (Tsiatsiani *et al.*, 2011; Van Doorn *et al.*, 2011). PCD has effectively been proved to occur in response to various abiotic stresses (Kadono *et al.*, 2010; Tran *et al.*, 2013b; Van Doorn *et al.*, 2011) comprising drought and salinity (Huh *et al.*, 2002).

1.6 Aim of this thesis

The aim of this thesis was to better understand the role of ion channels during ionic- and non-ionic hyperosmotic stress leading to Programmed Cell Death (PCD). In the first study, BY2-tobacco cells was choosen as cellular model study and *Arabidopsis thaliana* in the second and third. Cultured cells represent a good model system to study abiotic and/or biotic stresses, in fact they are characterized by high omogeneity and high growth rate. A.thaliana suspension cells allow working on mutants. Moreover, they are a good tool for studies at cellular levels comprising microelectrode voltage clamp. The microelectrode voltage clamp is a method that allows the measurements of ion flow through a membrane as an electrical current in intact cells, preserving their cell wall, unlike the patch clamp that requires the use of isolate protoplasts (Bouteau and Tran, 2012). The main objectives of this work were to compare and understand the role of the ion currents induced by saline and non-saline hyperosmotic stress leading to PCD and search for putative regulators of hyperosmotic-induced PCD in plants

Chapter 2

Early events involved in hyperosmotic stress induced PCD in tobacco BY2 cells

2.1 Introduction

Ionic and non-ionic hyperosmotic stresses have severe effects on plant growth (Tester and Davenport, 2003). Salt stress induces complex and wide series of cellular responses that may result in the induction of signalling events that lead to PCD (Huh *et al.*, 2002; Shabala, 2009). Also drought was shown to induce PCD (Duan *et al.*, 2010). In this study, by using BY2 cells, we compared the early events induced by ionic and non-ionic hyperosmotic stress and their relation(s) with ion channels regulation and PCD. Ion channels activity was recorded by using the microelectrodes voltage-clamps techniques (dSEVC). With this technique it's possible to measure transmembrane potential changes and ion channels activity from intact cultured cells preserving their cell wall. This is a point of great importance in plant cells, since the cell wall could not allow a volume increase as observed in animal cells (cf RVI, §1.4). The figure 19 shows a plant cell sustained by a microfunnel and impaled by a microelectrode allowing dSEVC recording.

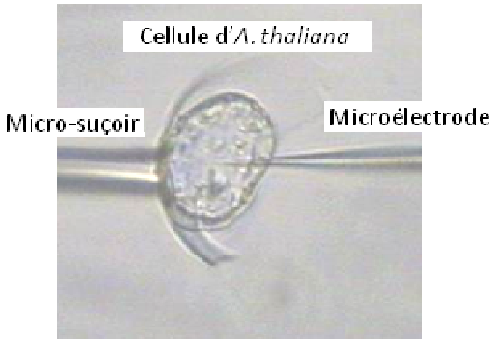

Figure 19: Typical st up of plant cells impalement for voltage clamp measurements.

In case of ionic hyperosmotic stress (addition of NaCl) it was possible to record plasma membrane depolarization due to influx of Na^+ and in case of non-ionic hyperosmotic stress (addition of sorbitol), we observed a hyperpolarization due to a decrease in anions currents. If a relation could be observed between Na^+ current and NaCl-induced PCD, no link was observed between anion current decrease and the sorbitol induced PCD.

2.2 Deciphering early events involved in hyperosmotic stress-induced programmed cell death in tobacco BY-2 cells

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RESEARCH PAPER

Deciphering early events involved in hyperosmotic stress-induced programmed cell death in tobacco BY-2 cells

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Abstract

Hyperosmotic stresses represent one of the major constraints that adversely affect plants growth, development, and productivity. In this study, the focus was on early responses to hyperosmotic stress- (NaCl and sorbitol) induced reactive oxygen species (ROS) generation, cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) increase, ion fluxes, and mitochondrial potential variations, and on their links in pathways leading to programmed cell death (PCD). By using BY-2 tobacco cells, it was shown that both NaCl- and sorbitol-induced PCD seemed to be dependent on superoxide anion ($O_2^{\cdot-}$) generation by NADPH-oxidase. In the case of NaCl, an early influx of sodium through non-selective cation channels participates in the development of PCD through mitochondrial dysfunction and NADPH-oxidase-dependent $O_2^{\cdot-}$ generation. This supports the hypothesis of different pathways in NaCl- and sorbitol-induced cell death. Surprisingly, other shared early responses, such as $[Ca^{2+}]_{cyt}$ increase and singlet oxygen production, do not seem to be involved in PCD.

Key words: Calcium, hyperosmotic stress, mitochondria, NaCl, *Nicotiana tabacum*, non-selective cation channels, programmed cell death, reactive oxygen species.

Introduction

Salt stress is known to have severe effects on plant growth and development (Tester and Davenport, 2003). High salinity leads to ionic, osmotic, and oxidative stress in plants (Zhu, 2001) that may result in the induction of signalling events that lead to programmed cell death (PCD) in higher plants (Huh *et al.*, 2002; Lin *et al.*, 2006; Shabala, 2009; Wang *et al.*, 2010) and algae (Affenzeller *et al.*, 2009). Such PCD could be regarded as a salt adaptation mechanism (Huh *et al.*, 2002). Drought, which consists at least in part of a hyperosmotic stress, was also shown to induce PCD in plants (Duan *et al.*, 2010). PCD is an active cellular process that facilitates the removal of unwanted or damaged cells and is essential for cellular differentiation and tissue homeostasis (van Doorn *et al.*, 2011). PCD has effectively been proved to occur in response

to various abiotic stresses (Kadono *et al.*, 2010; van Doorn *et al.*, 2011). Different types of PCD with overlapping morphological and physiological hallmarks have been described in plants, which has led to a call for a detailed classification of cell death events (van Doorn, 2011; van Doorn *et al.*, 2011). Although the delineation between the different PCD types of sometimes remains difficult (van Doorn *et al.*, 2011), typical hallmarks of PCD in plants frequently include the fragmentation of the DNA by specific nucleases (DNA laddering), condensation and shrinkage of the cytoplasm, release of cytochrome *c* from mitochondria, elevation of the cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$), generation of reactive oxygen species (ROS), and an activity increase of caspase-like enzymes (van Doorn, 2011; Tsiatsiani *et al.*, 2011).

Although early events reported in responses to ionic and non-ionic hyperosmotic stress such as a transient $[Ca^{2+}]_{cyt}$ increase (Xiong *et al.*, 2002; Donaldson *et al.*, 2004; Lin *et al.*, 2006; Kim *et al.*, 2007; Parre *et al.*, 2007; Ranf *et al.*, 2008), generation of ROS (Zhu, 2001; Xiong *et al.*, 2002; Lin *et al.*, 2006; Zhang *et al.* 2013), or up-regulation of protein kinases (Zhang *et al.*, 2013) seemed to be shared in plants, some other responses are specific to one of these stresses. Under saline conditions, Na^+ enters the cells through non-selective cation channels (NSCCs; Demidchik and Tester, 2002), depolarizing the plasma membrane (Chen *et al.*, 2007; Hua *et al.*, 2008; Shabala and Cuin, 2008; Pandolfi *et al.*, 2010; Wegner *et al.*, 2011). In contrast, isotonic mannitol or sorbitol solutions cause significant membrane hyperpolarization (Li and Delrot, 1987; Zingarelli *et al.*, 1999; Shabala *et al.*, 2000; Shabala and Lew, 2002). The DNA laddering, due to endonuclease release through permeable transition pores (PTPs) leading to mitochondria depolarization (Huh *et al.*, 2002; Lin *et al.*, 2006) occurred in NaCl- but not in sorbitol-stressed cells (Affenzeller *et al.*, 2009). Thus, although some common events are induced upon osmotic stress, multiple signal transduction pathways are involved in the response to ionic and non-ionic hyperosmotic treatments (Donaldson *et al.*, 2004; Parré *et al.*, 2007).

In this study, it was shown using Bright Yellow 2 (BY-2) cells that both ionic and non-ionic hyperosmotic stresses effectively induced early singlet oxygen ($^1O_2$) generation and an $^1O_2$-dependent influx of Ca^{2+}, which are not involved in PCD processes. The PCD observed in response to NaCl and sorbitol seemed to be dependent on delayed superoxide anion ($O_2^{\cdot-}$) generation by NADPH-oxidase, this last being linked to Na^+ influx through NSCCs and mitochondrial dysfunction only in the case of NaCl hyperosmotic stress.

Materials and methods

Cell culture conditions

Nicotiana tabacum L. BY-2 suspension cells were grown in Murashige and Skoog (MS) medium, pH 5.8 augmented with 30 g l^{-1} sucrose and 0.2 mg l^{-1} 2,4 D (Pauly *et al.*, 2001). Cells were maintained at 22 ± 2 °C, under continuous darkness and continuous shaking (gyratory shaker) at 120 rpm. Cell suspensions were subcultured weekly using a 1:15 dilution. All experiments were performed at 22 ± 2 °C using log-phase cells (6 d after subculture) maintained in their culture medium. Cell density was ~4×10^5 cells ml^{-1}.

Osmolality changes

The osmolality changes were systematically obtained by addition of 50 µl of sorbitol or NaCl from various stock solutions. For the measurement of extracellular medium osmolality changes after NaCl or sorbitol treatment, 100 µl of supernatant of cell suspensions treated with NaCl or sorbitol, were determined by the freezing depression method using an Automatic Micro-Osmometer Type 15 (Löser Messtechnik, Berlin, Germany).

Cell viability assays

Hyperosmosis-induced cell death in the cell suspension culture was determined by staining the dead cells with the vital dye Evans blue (0.005%, w/v) by mixing and incubating the cells and the dye for 10 min. Then stained cells were observed under a microscope. When appropriate, a 15 min pre-treatment with pharmacological effectors was done prior to NaCl or sorbitol exposure. Cells were counted under a microscope and cells that accumulated Evans blue were considered dead. At least 500 cells were counted for each independent treatment, and the procedure was repeated at least three times for each condition.

Monitoring of ROS production

The production of ROS was monitored by the chemiluminescence of the *Cypridina* luciferin analogue (CLA) as previously described (Kadono *et al.*, 2006, 2010). CLA is known to react mainly with $O_2^{\cdot-}$ and $^1O_2$ with light emission (Nakano, 1986), and allows measurement of extracellular ROS in plant cells (Tran *et al.*, 2013). Chemiluminescence from CLA was monitored using an FB12-Berthold luminometer (with a signal integrating time of 0.2 s). For data analysis, the luminescence ratio (L/L_{basal}) was calculated by dividing the intensity of CLA luminescence (L) by the luminescence intensity before treatment (L_{basal}). The ROS scavengers 1,2-dihydroxybenzene-3,5-disulphonic acid disodium salt (Tiron), 1,4-diazabicyclo[2.2.2]octane (DABCO), and salicylhydroxamic acid (SHAM) were added 5 min before NaCl and sorbitol treatment. Other inhibitors were added to the cell suspension 30 min before NaCl and sorbitol treatment.

Aequorin luminescence measurements

The $[Ca^{2+}]_{cyt}$ variations were recorded in a BY-2 cell suspension expressing the aequorin gene. Aequorin was reconstituted by overnight incubation in MS medium supplemented with 30 g l^{-1} sucrose and 2.5 µM native coelenterazine. Cell culture aliquots (500 µl) were transferred carefully into a luminometer tube, and the luminescence counts were recorded continuously at 0.2 s intervals with a luminometer. Treatments were performed by pipette injection of 50 µl of the effectors (NaCl or sorbitol). The residual aequorin was discharged by addition of 500 µl of a 1 M $CaCl_2$ solution dissolved in 100% methanol. The resulting luminescence was used to estimate the total amount of aequorin in each experiment. Calibration of calcium measurement was performed by using the equation: $pCa=0.332588(-\log k)+5.5593$, where k is a rate constant equal to luminescence counts per second divided by the total remaining counts (Knight *et al.*, 1996). The results are expressed in micromolar Ca^{2+} and correspond to the mean±SD of 3–5 independent experiments.

Voltage clamp measurements

Experiments were conducted on 6-day-old cells maintained in their culture medium to limit stress (main ions in MS medium 28 mM NO_3^-, 16 mM K^+). Individual cells were immobilized by a microfunnel (~50–80 µm outer diameter) and controlled by a micromanipulator (WR6-1, Narishige, Japan). Impalements were carried out with a piezoelectric micromanipulator (PCS-5000, Burleigh Inst., USA) in

Chapter 2 Early events involved in hyperosmotic stress induced PCD in tobacco BY2 cells

a chamber (500 µl) made of Perspex. Voltage clamp measurements of whole-cell currents from intact cultured cells presenting a stable running membrane potential were carried out using the technique of the discontinuous single voltage clamp microelectrode (dSEVC; Finkel and Redman, 1984). In this technique, both current passing and voltage recording use the same microelectrode. Interactions between the two tasks are prevented by time-sharing techniques (sampling frequency 1.5–3 kHz). Microelectrodes were made from borosilicate capillary glass (Clark GC 150F, Clark Electromedical, Pangbourne Reading, UK) pulled on a vertical puller (Narishige PEII, Japan). Their tips were <1 µm diameter; they were filled with 600 mM KCl, and had electrical resistances between 20 MΩ and 50 MΩ with the culture medium. The capacity compensation of the microelectrode amplifier (Axoclamp 2A, Molecular Devices, Sunnyvale, CA, USA) was set to a subcritical level to produce the fastest electrode response. The relatively large size of the cells ensured a sufficiently high membrane time constant despite a relatively low input resistance (~40 MΩ). Specific software (pCLAMP 8) drives the voltage clamp amplifier. Voltage and current were simultaneously displayed on a dual input oscilloscope (Gould 1425, Gould Instruments Ltd, Hainault, UK), digitalized with a Digidata 1322A (Molecular Devices). In whole-cell current measurements, the membrane potential was held to the value of the resting membrane potential. Current recordings were obtained by hyperpolarizing pulses from –200 mV to +80 mV (20 mV, 2 s steps of current injection, 6 s of settling time). It was systematically checked that cells were correctly clamped by comparing the protocol voltage values with those really imposed. Only microelectrodes presenting a linear relationship were used.

Confocal microscopy

Confocal imaging was performed using an upright Leica Laser Scanning Confocal Microscope SP5 (Leica Microsystems, Germany) equipped with a ×63 oil immersion objective. To analyse the NaCl influx, the sodium indicator Sodium Green was used (Molecular Probes, USA). The BY-2 tobacco cells were pre-incubated for 15 min with an NSCC inhibitor and then incubated with 200 mM NaCl for 1 h. Sodium Green indicator (10 µM) was added to the solution 30 min after the beginning of the salt treatment. After incubation with NaCl and Sodium Green indicator, the BY-2 cells were washed with phosphate-buffered saline (PBS) buffer. The excitation wavelength was set at 514 nm, and the emission was detected at 530 ± 20 nm.

Mitochondrial membrane potential measurement

Six-day-old BY-2 suspension cells were collected and washed by filtration in a suspension buffer containing 50 mM HEPES, 0.5 mM $CaCl_2$, 0.5 mM K_2SO_4, and 10 mM glucose, pH 7.0 (Errakhi *et al.*, 2008). After treatment, cells were stained with the mitochondrial membrane potential probe JC-1 by incubating 2 ml of cell suspensions for 15 min (24 °C in the dark) with 2 µg ml^{-1} JC-1 (3 µM). JC-1 was dissolved and stored according to the manufacturer's instructions. Treated cells without prior washing were subjected to analysis using a Hitachi F-2000 fluorescence spectrophotometer. The excitation wavelength used was 500 nm. Fluorescence signals were collected using a band pass filter centred at 530 nm and 590 nm.

Chemicals

All chemical products were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France), except JC1 and Sodium Green indicator which were from Molecular Probes (Saint Aubin, France). Stock solution of diphenyleneiodonium chloride (DPI; 10 mM) was dissolved in dimethylsulphoxide (DMSO) in order to obtain a 0.01% final concentration of DMSO. This DMSO concentration did not induce any change in ROS or $[Ca^{2+}]_{cyt}$ levels (not shown). All other chemicals were dissolved in water.

Statistical analysis

Data were analysed by analysis of variance (ANOVA), and the mean separation was achieved by Newman and Keuls multiple range test. All numerical differences in the data were considered significantly different at the probability level of $P \leq 0.05$.

Results

Hyperosmotic changes induce cell death in N. tabacum *BY-2 suspension-cultured cells*

The impact of NaCl and sorbitol additions on osmolality changes in BY-2 medium was first evaluated and it was found that the concentrations of NaCl (200 mM) and sorbitol (400 mM) most frequently used in this study showed almost the same osmolality shifts (Table 1). These shifts in osmolality induced by 400 mM sorbitol or 200 mM NaCl led to the death of a part of the cell population, dead cells displaying large cell shrinkage (Fig. 1A), the hallmark of the PCD process (van Doorn, 2011). Cell death scoring at various concentrations of sorbitol and NaCl showed the time- and dose-dependent progression of death (Fig. 1B, C), half of the cells being dead after 4 h at 400 mM sorbitol and 200 mM NaCl. In order to confirm whether this cell death was due to an active process requiring active gene expression and cellular metabolism, BY-2 cell suspensions were treated with actinomycin D (AD), an inhibitor of RNA synthesis, or with cycloheximide (Chx), an inhibitor of protein synthesis, each at 20 mg ml^{-1}, 15 min prior to 200 mM NaCl or 400 mM sorbitol exposure. In both cases, AD and Chx significantly reduced cell death (Fig. 1D). These results indicated that this cell death required active cell metabolism, namely gene transcription and *de novo* protein synthesis. Taken together, these data showed that saline or non-saline hyperosmotic stress induced a rapid PCD of a part of the *N. tabacum* BY-2 suspension cell population.

The kinetics of some early events classically detected upon saline stress or drought, namely an increase in cytosolic Ca^{2+}, ion flux variations, ROS production, and mitochondrial membrane depolarization, were then followed, and it was checked how they could be involved in PCD induced by hyperosmotic stress.

Sorbitol- and NaCl-induced ROS generation

To study the effect of sorbitol on production of ROS in BY-2 cell suspension culture, the chemiluminescence of CLA, which indicates the generation of $O_2^{\cdot -}$ and $^1O_2$, was

Table 1. *Osmolality changes in the medium after treatment with NaCl and sorbitol*

	Medium	NaCl (mM)			Sorbitol (mM)		
		100	200	300	200	400	600
Osmolality (mosmol)	182	369	558	944	375	605	952

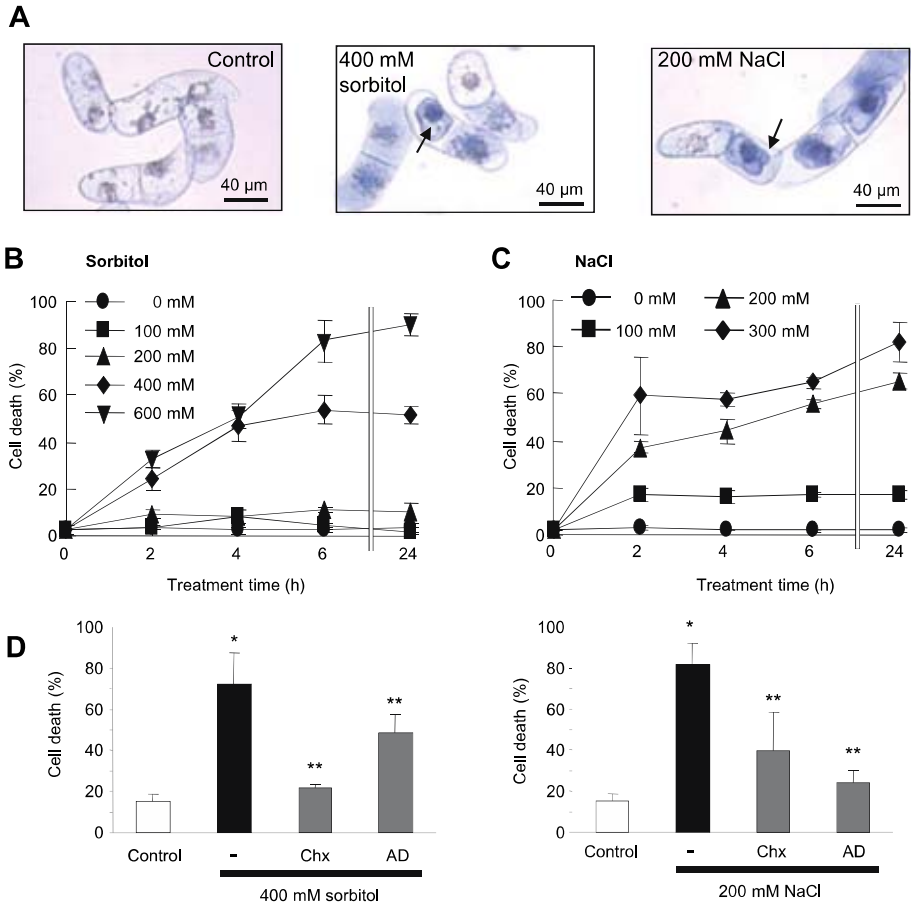

Fig. 1. NaCl- and sorbitol-induced cell death in tobacco BY-2 cells. (A) Light micrographs of BY-2 cultured cells stained with Evans blue 2 h after incubation with 400 mM sorbitol (centre) or 200 mM NaCl (right) compared with control cells maintained in their medium (left). Arrows indicate the cell shrinkage. (B) Effect of incubation time and concentration of sorbitol or NaCl on the extent of cell death. (C) Effect of pre-treatment with actinomycin D (AD; 20 µg ml^{-1}) or cycloheximide (Chx; 20 µg ml^{-1}) on sorbitol- and NaCl-induced cell death. Each data point and error bar reflect the mean and SD, respectively, of at least three independent replicates. *Significantly different from controls, $P < 0.05$; **significantly different from the NaC-l or sorbitol-treated cells, $P < 0.05$. (This figure is available in colour at *JXB* online.)

used. Addition of 400 mM sorbitol to BY-2 cell suspension culture resulted in transient production of ROS that reaches the maximal level immediately after treatment (Fig. 2A). This sorbitol-induced ROS generation was dose dependent (Fig. 2B) and could be blocked using DABCO, an $^1O_2$ scavenger, but not Tiron, an $O_2^{\cdot-}$ scavenger (Fig. 2A, C). Addition of 200 mM NaCl to BY-2 cell suspension culture also resulted in transient production of ROS that reaches the maximal level immediately after NaCl treatment (Fig. 2D, E). In the case of sorbitol, only DABCO was able to decrease the NaCl-induced CLA chemiluminescence (Fig. 2D, F). Thus, in both cases the early increase in CLA chemiluminescence seemed to be dependent on $^1O_2$ generation but not on $O_2^{\cdot-}$ generation. SHAM, an inhibitor of peroxidase (POX) (Kawano *et al.*, 1998; Hossain *et al.*, 2013), which could be responsible for extracellular $^1O_2$ generation (Kawano *et al.*, 1998; Kanofsky, 2000; Guo *et al.*, 2009), was thus used. Pre-treatment of the BY-2 cell suspension culture with 5 mM SHAM only slightly reduced the increase in CLA chemiluminescence induced by 400 mM sorbitol (Fig. 2C) but significantly reduced that induced by 200 mM NaCl (Fig. 2F). This suggests the involvement of POX, at least in NaCl-induced $^1O_2$ generation.

The impact of ROS pharmacology on NaCl- and sorbitol-induced PCD (Fig. 1) was further checked. DABCO, the $^1O_2$ scavenger, failed to decrease sorbitol- (400 mM) and

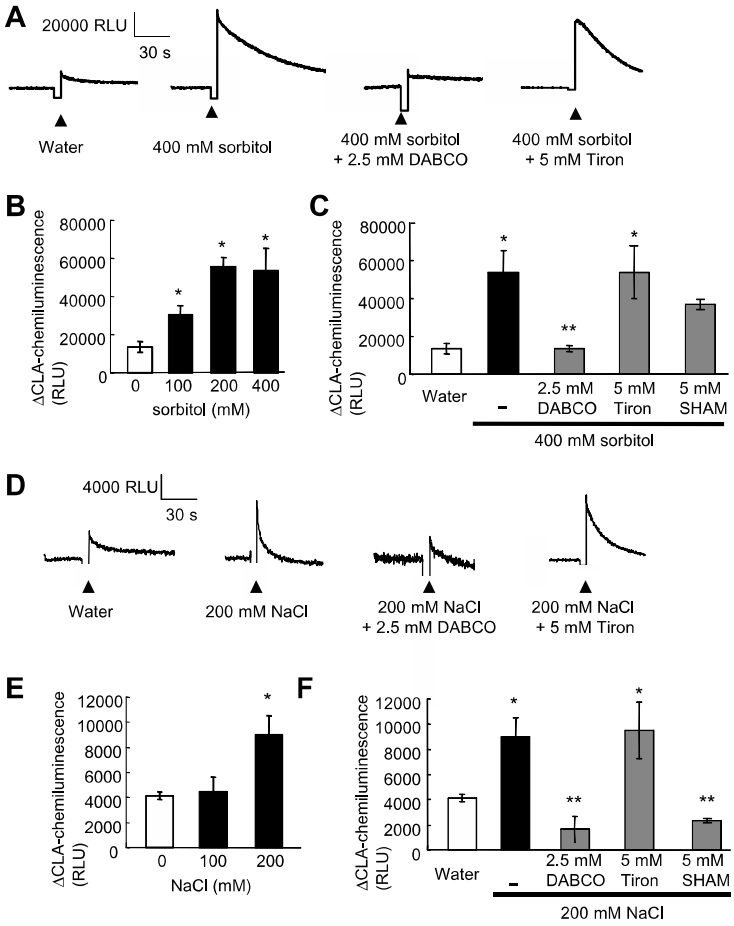

Fig. 2. Induction of rapid ROS generation in tobacco BY-2 cells by sorbitol or NaCl. (A) Typical kinetics of the sorbitol-induced increase in CLA chemiluminescence reflecting the production ROS and modulation by ROS scavengers. (B) Effect of the concentration of sorbitol on ROS generation. (C) Modulation of sorbitol-induced ROS generation by DABCO, a scavenger of singlet oxygen, Tiron, a scavenger of the superoxide anion, or salicylhydroxamic acid (SHAM), an inhibitor of peroxidase. (D) Typical kinetics of the NaCl-induced increase in CLA chemiluminescence and modulation by ROS scavengers. (E) Effect of the concentration of NaCl on ROS generation. (F) Modulation of NaCl-induced ROS generation by DABCO, Tiron, or SHAM. Each data point and error bar reflect the mean and SD, respectively (n=5). *Significantly different from controls, $P < 0.05$; **significantly different from the NaCl- or sorbitol-treated cells, $P < 0.05$.

NaCl- (200 mM) induced cell death and even increased NaCl-induced cell death after 2 h of treatment (Fig. 3A, B). For Tiron, the $O_2^{\cdot -}$ scavenger, there was no effect after 2 h but a decrease in cell death could be observed after 4 h treatment with NaCl (Fig. 3B). Thus, the hyperosmotic stress-induced cell death seemed not to be dependent on $^1O_2$ generation but on $O_2^{\cdot -}$ generation. Thus a possible delayed $O_2^{\cdot -}$ generation after treatment with 400 mM sorbitol or 200 mM NaCl was searched for. After such hyperosmotic stress, increases in CLA chemiluminescence could be detected (Fig. 3C). In both cases of CLA chemiluminescence increases, the maximum chemiluminescence levels were reached after 1 h and then decreased to the control level after 4 h, the decrease being more rapid upon NaCl treatement (Fig. 3C). It is noteworthy that these increases occurred before cell death reached the plateau level (Fig. 1B, C). These increases in CLA chemiluminescence could be inhibited by pre-treatment with Tiron, but also with 10 μM DPI, an inhibitor of NADPH-oxidase (Fig. 3D), suggesting that the generation $O_2^{\cdot -}$ through enhancement of NADPH-oxidase activity was involved in the delayed ROS generation after treatment with sorbitol and NaCl (Fig. 3C). In agreement with this, 10 μM DPI could also significantly reduce both sorbitol- and NaCl-induced cell death after 4 h (Fig. 3A, B).

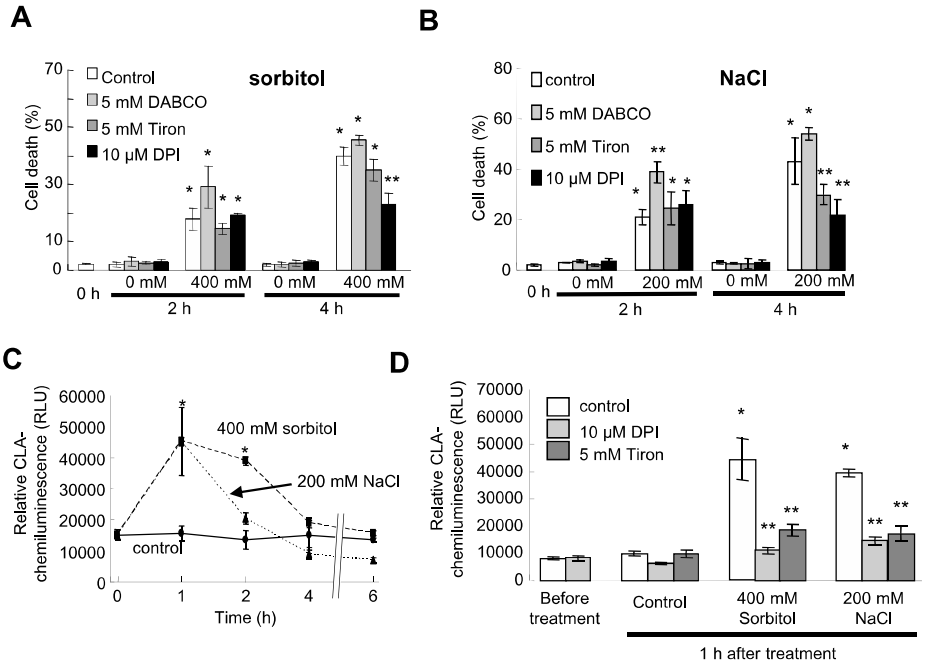

Fig. 3. Effects of ROS scavengers on NaCl- or sorbitol-induced cell death in BY-2 cells. (A) Effect of Tiron, DABCO, or DPI, an NADPH-oxidase inhibitor, on cell death induced by 400 mM sorbitol after 2 h or 4 h treatment. (B) Effect of Tiron, DABCO, or DPI on cell death induced by 200 mM NaCl after 2 h or 4 h treatment. (C) Time course of CLA chemiluminescence during 6 h treatment with 400 mM sorbitol or 200 mM NaCl. (D) Inhibition of sorbitol and NaCl-induced delayed ROS generation by Tiron or DPI. Each data point and error bar reflect the mean and SD, respectively (n=3). *Significantly different from controls, $P < 0.05$; **significantly different from the NaCl- or sorbitol-treated cells, $P < 0.05$.

Sorbitol and NaCl induce a rapid change in $[Ca^{2+}]_{cyt}$

The changes in $[Ca^{2+}]_{cyt}$ were monitored by the Ca^{2+}-dependent emission of blue light from aequorin (Knight *et al.*, 1996). Treatment of BY-2 cells with sorbitol (400 mM) resulted in a rapid transient increase in aequorin luminescence (Fig. 4A) reflecting an increase in $[Ca^{2+}]_{cyt}$ of 0.145 ± 0.035 μM (n=23). This increase could be inhibited by the presence of Ca^{2+} channel blockers, $LaCl_3$ and $GdCl_3$, when Ca^{2+} internal store inhibitors (U73122 and dantrolene, Meimoun *et al.*, 2009) showed no significant inhibitory effects (Fig. 4B), indicating that the sorbitol-induced increase in $[Ca^{2+}]_{cyt}$ is mainly due to influx of Ca^{2+} across the plasma membrane through Ca^{2+} channels. As the generation of $^1O_2$ by sorbitol occurred immediately upon hyperosmotic stress (Fig. 2A), the effect of ROS pharmacology on the sorbitol-induced increase in $[Ca^{2+}]_{cyt}$ was further tested. The $^1O_2$ scavenger DABCO strongly reduced the $[Ca^{2+}]_{cyt}$ increase compared with that in control cells; the POX inhibitor SHAM showed lower efficiency, while no differences were seen with the $O_2^{\cdot -}$ scavenger Tiron (Fig. 4A, C). These results strongly suggested that sorbitol-induced Ca^{2+} uptake through Ca^{2+} channels occurs downstream of the sorbitol-induced $^1O_2$ generation.

In the same way, treatment of BY-2 cells with NaCl (200 mM) resulted in a transient increase in aequorin luminescence (Fig. 4D), reflecting an increase in $[Ca^{2+}]_{cyt}$ of 0.217 ± 0.059 μM (n=23). As for sorbitol, inhibitors of Ca^{2+} release from intracellular organelles (U73122 and dantrolene) failed to suppress the NaCl-induced increase in $[Ca^{2+}]_{cyt}$ whereas Ca^{2+} channel blockers $LaCl_3$ and $GdCl_3$ were efficient at reducing the NaCl-induced increase in $[Ca^{2+}]_{cyt}$ (Fig. 4E). In a similar manner to what was observed for sorbitol, this increase was shown to be inhibited by the presence of DABCO (Fig. 4F) but less efficiently by SHAM, whereas Tiron seemed even to increase this Ca^{2+} influx (Fig. 4D, F). The effect of the Ca^{2+} channel inhibitor La^{3+} on NaCl- and sorbitol-induced cell death in BY-2 suspension-cultured cells was then studied. Lanthanum (500 μM) failed to decrease sorbitol- (400 mM) and NaCl- (200 mM) induced cell death and even increases this cell death after 2 h of treatment (Fig. 4G), as observed for DABCO with NaCl (Fig. 3B). These data are in agreement with the link observed between the immediate $^1O_2$ generation inducing an influx of Ca^{2+} upon sorbitol or NaCl stress and further suggest that these early induced events are not involved in a pathway leading to PCD.

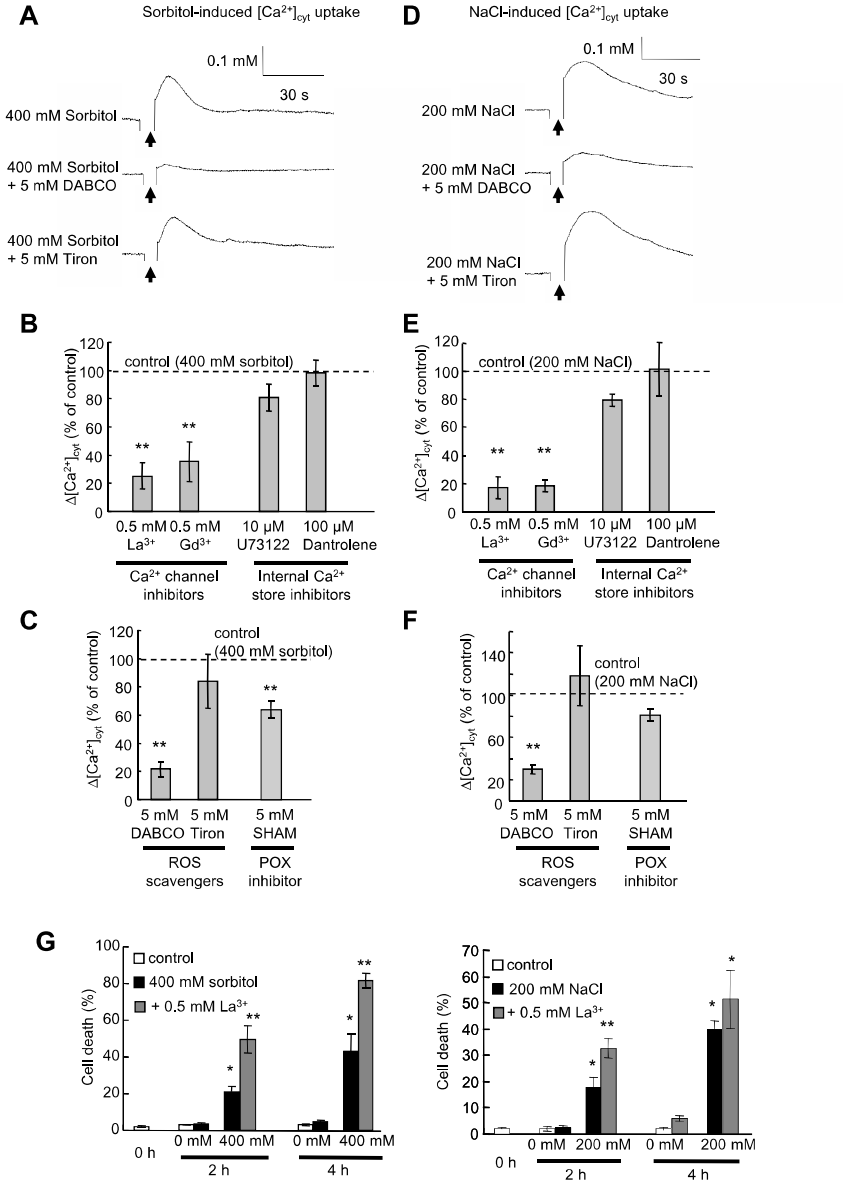

Fig. 4. Induction of $[Ca^{2+}]_{cyt}$ increase in aequorin-expressing tobacco BY-2 cells by sorbitol or NaCl. (A) Typical kinetics of sorbitol-induced increase in $[Ca^{2+}]_{cyt}$ and modulation by ROS scavengers. (B) Modulation of sorbitol-induced $[Ca^{2+}]_{cyt}$ increase by the calcium channel blockers La^{3+} or Gd^{3+} (500 μM each) and the internal stock release inhibitors U73122 (100 μM) and dantrolene (10 μM). (C) Modulation of sorbitol-induced $[Ca^{2+}]_{cyt}$ increase by DABCO, a scavenger of singlet oxygen, Tiron, a scavenger of anion superoxide, or salicylhydroxamic acid (SHAM), an inhibitor of peroxidase. (D) Typical kinetics of the NaCl-induced increase in $[Ca^{2+}]_{cyt}$ and modulation by ROS scavengers. (E) Modulation of the NaCl-induced $[Ca^{2+}]_{cyt}$ increase by La^{3+}, Gd^{3+} (500 μM each), U73122 (100 μM), or dantrolene (10 μM). (F) Modulation of NaCl-induced $[Ca^{2+}]_{cyt}$ increase by DABCO, Tiron, or SHAM. (G) Effect of La^{3+} on cell death induced by 200 mM NaCl (left) or 400 mM sorbitol (right) after 2 h or 4 h treatment. Each data point and error bar reflect the mean and SD, respectively (n=5). *Significantly different from controls, $P < 0.05$; **significantly different from the NaCl- or sorbitol-treated cells, $P < 0.05$.

Hyperosmotic constraints induce change in membrane potential and ion channel activities

Saline and non-saline hyperosmotic stresses are well known to modify the plasma membrane potential (V_m) of cells (Teodoro *et al.*, 1998; Zingarelli *et al.*, 1999; Shabala and Cuin, 2008; Wegner *et al.*, 2011). By using an electrophysiological technique (dSEVC), the impact of NaCl and sorbitol on BY-2 cultured cell membrane potential was investigated. In control conditions in culture medium, the V_m of BY-2 cells was –21.1 ± 2.2 mV (n=15). In MS medium, the main ions are 16 mM K^+ and 28 mM NO_3^-; thus the equilibrium potential estimated for K^+, E_K, is about –46 mV ($[K^+]_{out}$=16 mM with $[K^+]_{in}$ estimated at 100 mM). The equilibrium potential estimated for NO_3^- is about –25 mV ($[NO_3^-]_{out}$=28 mM with $[NO_3^-]_{in}$ estimated at 5 mM). As previously observed with cultured cells of *Arabidopsis thaliana* (Kadono *et al.*, 2010; Tran *et al.*, 2013) or tobacco (Gauthier *et al.*, 2007), the occurrence of anion currents in most of the BY-2 cells in their culture medium could explain the mean polarization of around –20 mV recorded in control and non-stressed conditions. The mean control value of these currents at –200 mV and after 1.8 s of voltage pulse was –1.12 ± 0.2 nA (n=11). These currents were shown to be sensitive to structurally unrelated anion channel inhibitors, 9-anthracene carboxylic acid (9-AC) and glibenclamide (gli) (Supplementary Fig. S1 available at *JXB* online), reinforcing the hypothesis of an anionic nature for these currents. Addition of NaCl to suspension cultures resulted in a significant membrane depolarization (Fig. 5A) when sorbitol induced a hyperpolarization of the cells (Fig. 5A), clearly indicating a difference between saline and non-saline hyperosmotic stress. The sorbitol-induced hyperpolarization was correlated with a decrease in anion current (Fig. 5B) when the NaCl-induced depolarization was correlated with a large increase in whole-cell ion current (Fig. 5B). The positive shifts of the reversal potential of the current upon addition of NaCl are in accordance with a current carried by Na^+.

The influx of Na^+ through the plasma membrane by NSCCs was the most probable reason for the cell depolarization (Demidchick *et al.*, 2002*a*, *b*), this Na^+ uptake was further checked using the Na^+-sensitive fluorescent probe Sodium Green (Wegner *et al.*, 2011). An accumulation of fluorescence in the cytoplasm of the cells could be observed after

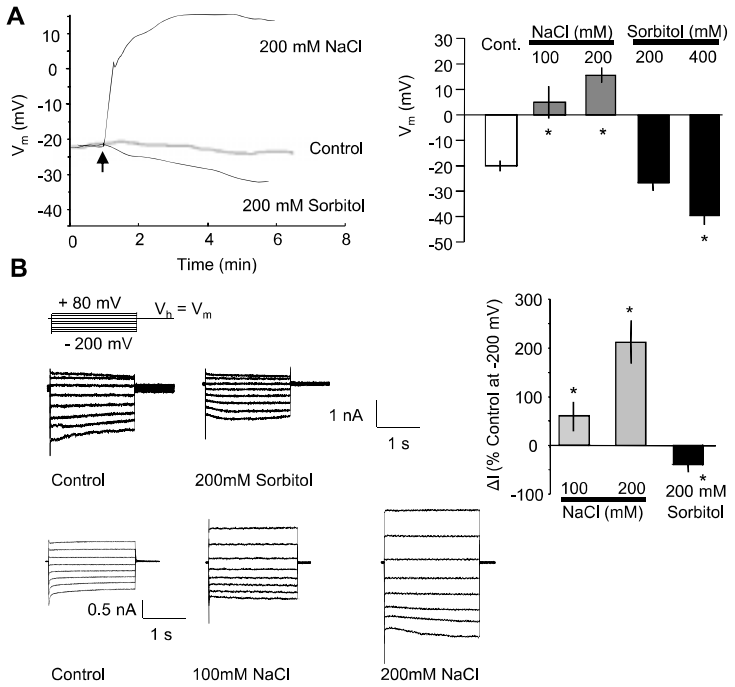

Fig. 5. (A) Typical modulation of BY-2 cultured cell plasma membrane (PM) potential variations observed in response to NaCl or sorbitol. (B) Mean values of PM potentials recorded a few minutes after treatment with NaCl (100 mM or 200 mM) or sorbitol (200 mM or 400 mM). (C) Typical changes in whole-cell current profiles after treatments with sorbitol (up) or NaCl (down). The protocol was as illustrated; the holding potential (V_h) was V_m. (D) Mean values of whole-cell current variations (recorded at –200 mV and 1.8 s) after treatment with NaCl or sorbitol. Current variations are given as a percentage of the control level before treatments. The data correspond to means of at least five independent replicates, and error bars correspond to the SD. *Significantly different from controls, $P < 0.05$.

1 h treatment with 200 mM NaCl (Fig. 6A). This fluorescence could be reduced by using pre-treatments with inhibitors of NSCC, 5 mM tetraethylammonium chloride (TEA^+) or 1 mM quinine (Demidchick *et al.*, 2002*a*) (Fig. 6A). Addition of 1 mM quinine or 5 mM TEA^+ also allowed reduction of the NaCl-induced increase in currents (Fig. 6B, C) in BY-2 cells, as did verapamil (100 µM), another potent inhibitor of NSCCs (Demidchick *et al.*, 2002*a*). The impact of these NSCC blockers on the extent of NaCl-induced cell death was thus further tested. The NSCC blockers were efficient at reducing the cell death induced by 200 mM NaCl (Fig. 6D), suggesting that NSCC activation is related to the NaCl-induced cell death. As this cell death was also dependent on a delayed $O_2^{\cdot-}$ generation by NADPH-oxidase (Fig. 3), the effect of the earliest activation of NSCCs by NaCl on this delayed ROS production was also checked. As for cell death, the NSCC blockers were efficient at reducing this NaCl-dependent delayed ROS production (Fig. 6E), suggesting that NaCl influx participates in the $O_2^{\cdot-}$ generation. Interestingly, the calcium channel blocker La^{3+} did not allow the delayed ROS generation to be decreased, confirming the hypothesis of the induction of different pathways in response to NaCl stress.

Mitochondrial depolarization is involved in NaCl- but not sorbitol-induced cell death

The role of mitochondria is well recognized in salinity tolerance (Jacoby *et al.*, 2011), and dysfunction of mitochondria, leading to cytochrome *c* release upon salt stress, was shown to induce PCD in *Thellungiella halophila* suspension-cultured cells (Wang *et al.*, 2010). Mitochondria are effectively pivotal in controlling cell life and PCD, through complex mechanisms that culminate in opening of PTPs leading to mitochondrial membrane potential ($\Delta\Psi_m$) loss (Vianello *et al.*, 2007). It was thus checked whether NaCl and sorbitol lead to a decrease in $\Delta\Psi_m$ in the present model. In untreated cells, the JC-1 fluorescence ratio of mitochondria displaying a high $\Delta\Psi_m$ versus mitochondria presenting a low $\Delta\Psi_m$ was greatly superior to 1 (Fig. 7A). This ratio decreased in a time-dependent manner upon addition of NaCl (200 mM), indicating that NaCl induced a significant decrease of $\Delta\Psi_m$ in some mitochondria when sorbitol (400 mM) induced an increase of $\Delta\Psi_m$ during the first 30 min, the ratio reaching the control value after 2 h (Fig. 7A). Since a mitochondrial $\Delta\Psi_m$ decrease during cell death was reported to be due to the formation of the mitochondrial PTPs (Vianello *et al.*, 2007), the effect of cyclosporin A (CsA), an inhibitor of PTPs, was tested on NaCl-induced mitochondrial depolarization. A pre-treatment with CsA significantly reduced the NaCl-induced mitochondrial depolarization after 15 min (Fig. 7B), indicating that mitochondrial PTPs were involved in NaCl-induced mitochondrial depolarization. Moreover, pre-treatment with CsA significantly inhibited NaCl-induced cell death (Fig. 7C), indicating that PTP formation could participate in NaCl-induced cell death. As the activation of NSCCs occurs rapidly upon NaCl stress (Fig. 5A) and is involved in ROS generation (Fig. 6E), the impact of sodium influx on $\Delta\Psi_m$ was investigated by using NSCC inhibitors. A significant reduction of NaCl-induced mitochondrial depolarization was observed after pre-treatment with verapamil (Fig. 7B). These data show that (i) two different pathways could be involved in the hyperosmotic stress-induced cell death; and (ii) the ROS generation dependent on Na^+ influx could participate in the decrease in $\Delta\Psi_m$ during NaCl-induced cell death.

Discussion

As expected from previous studies (Huh, 2002; Wang, 2010), the death of a part of the BY-2 cell population characterized by large cell shrinkage, a hallmark of the PCD process (van Doorn, 2011), was observed in response to NaCl-induced hyperosmotic stress. The extent of cell death was time and dose dependent, reaching about half of the population in 4 h with 200 mM NaCl. In order to check whether this cell death was due to an active mechanism requiring active gene expression and cellular metabolism, BY-2 cell suspensions were treated with AD, an inhibitor of RNA synthesis, or with Chx, an inhibitor of protein synthesis, prior to NaCl exposure. AD and Chx significantly reduced the NaCl-induced cell death. These results indicated that this cell death required active cell metabolism, namely gene transcription and *de novo* protein synthesis. The same behavior, namely time- and dose-dependent cell death characterized by cell shrinkage and requiring active metabolism using iso-osmotic concentrations of sorbitol, was observed here. This suggests that non-ionic hyperosmotic stress, like ionic hyperosmotic stress, could induce PCD in BY-2 cells as previously observed in various animal cell lines (Murata *et al.*, 2002; Galvez *et al.*, 2003; Niswander and Dokas, 2007). It was then checked whether some early events classically detected during hyperosmotic stress responses and PCD in plant could be involved in this cell death, namely ROS production, an increase in cytosolic Ca^{2+}, ion flux variations, and mitochondrial membrane depolarization.

The earliest response observed after exposure of BY-2 cells to NaCl was an immediate peak of ROS. Addition of Tiron (a scavenger of $O_2^{\cdot-}$) did not significantly reduce NaCl-induced ROS generation, whereas DABCO (a scavenger of $^1O_2$) avoided this production. Although $^1O_2$ formation is likely to occur during the exposure to high light intensities (Krieger-Liszkay, 2004), it was also found that different enzymes including POXs could produce extracellular $^1O_2$ in animals (Kanofsky, 2000; Stief, 2003; Tarr and Valenzeno, 2003) and in plant cells (Kawano *et al.*, 1998; Guo *et al.*, 2009). As the studied cultured cells were non-photosynthetic, the POX inhibitor SHAM was further tested (Kawano *et al.*, 1998; Hossain *et al.*, 2013), and this could reduce the early NaCl-induced ROS generation effectively. Thus, it would appear that these early transient $^1O_2$ generations are not specific to ionic or non-ionic hyperosmotic stress.

In the case of both NaCl and sorbitol treatments, rapid transient $[Ca^{2+}]_{cyt}$ increases in BY-2 cells were observed, as previously described in various models (Knight *et al.*, 1997; Donaldson *et al.*, 2004; Lin *et al.*, 2006; Parre *et al.*, 2007; Ranf *et al.*, 2008). In BY-2 cells, these $[Ca^{2+}]_{cyt}$ increases were inhibited by the calcium channel blockers La^{3+} and

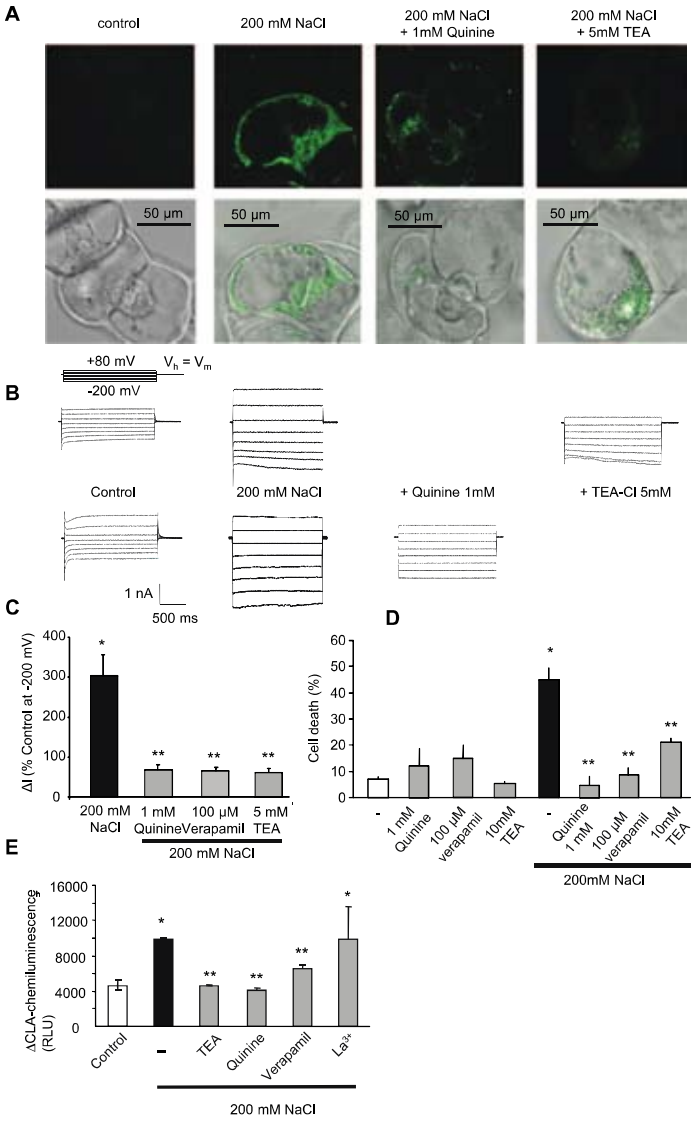

Fig. 6. (A) Confocal imaging of NaCl accumulation in BY-2 cells after 1 h treatment with 200 mM NaCl using the Sodium Green fluorescent probe (left). Decrease in fluorescence in cells pre-treated with quinine (1 mM) or TEA^+ (5 mM), blockers of non-selective cation channels (NSCCs), prior to NaCl treatment (right). Corresponding bright field images are shown on the line below. Each image is representative of symptoms observed in at least three independent experiments. (B) Variations of whole-cell currents recorded before and after addition of 200 mM NaCl and subsequent addition of 1 mM quinine or 5 mM TEA^+. The protocol was as illustrated; the holding potential (V_h) was V_m. (C) Mean values of whole-cell current variations (recorded at –200 mV and 1.8 s) after treatment with 200 mM NaCl with or without the NSCC blockers quinine (1 mM), TEA^+ (5 mM), or verapamil (200 µM). Current variations are given as a percentage of the control level before treatments. The data correspond to means of at least five independent replicates, and error bars correspond to the SD. (D) Effect of pre-treatments with the NSCC blockers on NaCl-induced cell death. The data correspond to means of at least four independent replicates, and error bars correspond to the SD. (E) Effect of pre-treatments with the NSCC blockers or with La^{3+} (500 µM) on NaCl-induced delayed ROS generation and cell death. The data correspond to means of at least five independent replicates, and error bars correspond to the SD. *Significantly different from controls, $P < 0.05$; **significantly different from the NaCl- or sorbitol-treated cells, $P < 0.05$. (This figure is available in colour at *JXB* online.)

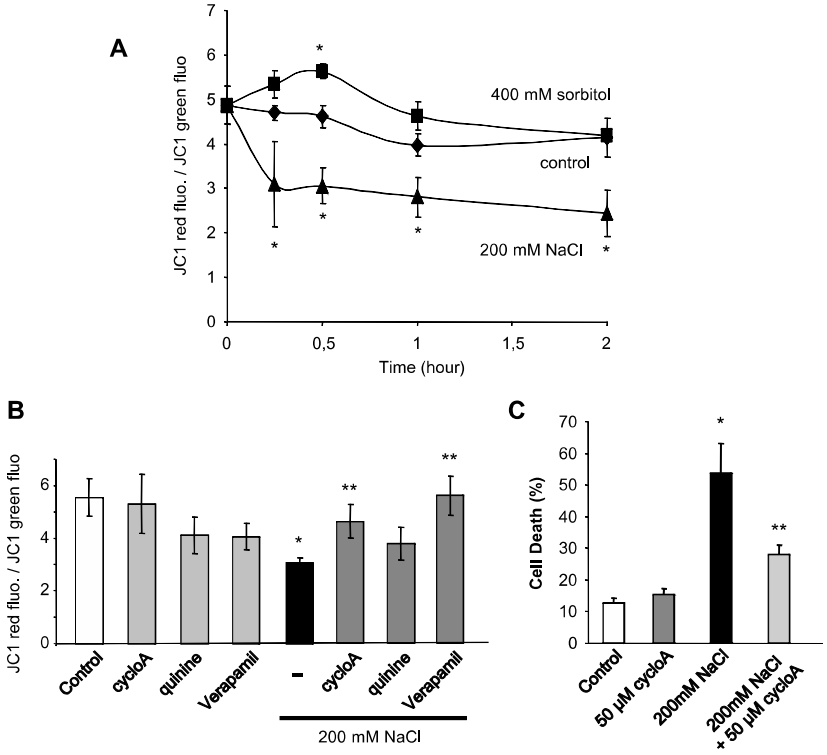

Fig. 7. (A) Variations of mitochondrial membrane potential ($\Delta\Psi_m$) of BY-2 tobacco cells after treatments with 200 mM NaCl or 400 mM sorbitol. (B) Effect of 50 µM cyclosporin A (CsA), an inhibitor of the permeability transition pore, 1 mM quinine, or 200 µM verapamil, inhibitors of NSCCs, on $\Delta\Psi_m$ variation induced by 200 mM NaCl after 15 min. The data reflect the means±SE of at least four independent experiments. (C) Effect of 50 µM CsA, 1 mM quinine, or 200 µM verapamil on cell death induced by 200 mM NaCl. The data reflect the means±SE of at least three independent replicates. *Significantly different from controls, $P < 0.05$; **significantly different from the NaCl-treated cells, $P < 0.05$.

Gd^{3+} but not by dantrolene and U73122, inhibitors of calcium-induced calcium release channels and of the inositol triphosphate receptor, respectively, known to be efficient in plants (Meimoun *et al.*, 2009). This suggests that the $[Ca^{2+}]_{cyt}$ increase was not due to Ca^{2+} release from intracellular Ca^{2+} stores, but to an influx through plasma membrane-permeable Ca^{2+} channels. Although rapid, the Ca^{2+} influxes happened after the $^1O_2$ generation since DABCO pre-treatments strongly decreased these influxes, suggesting that they were dependent on the generation of $^1O_2$. Accordingly, SHAM decreased the Ca^{2+} influxes to a lower extent and Tiron was unable to decrease them. This confirms the hypothesis that an $^1O_2$-dependent Ca^{2+} influx was induced by sorbitol and NaCl and highlights the role of $^1O_2$ as a signalling molecule (Fischer *et al.*, 2013).

Perturbation of Ca^{2+} homeostasis in plant cells, as well as in animal cells, has been described as a prerequisite for PCD (Grant *et al.*, 2000; Davis and Distelhorst, 2006; Lecourieux *et al.*, 2006). It was not possible to ascertain that in the BY-2 population all cells respond with a Ca^{2+} increase in the face of hyperosmotic stress. However, if only non-dying cells respond with a Ca^{2+} increase, this Ca^{2+} increase has no role in inducing PCD. In other words, if only dying cells (or even all cells) respond with a Ca^{2+} increase, since La^{3+} was inefficient in decreasing NaCl- and sorbitol-induced cell death, and even enhanced this cell death, it could suggest that Ca^{2+} influx could participate in cell protection. After perception of salt stress, the Ca^{2+} spike generated in the cytoplasm of root cells is known to activate the *S*alt *O*verly *S*ensitive (SOS) signal transduction cascade to protect the cells from damage due to excessive ion accumulation (Ji *et al.*, 2013). *SOS3* encodes a myristoylated calcium-binding protein that appears to function as a primary calcium sensor to perceive the increase in cytosolic Ca^{2+} triggered by Na^+ excess that has entered the cytoplasm. Upon binding to Ca^{2+}, SOS3 is able to interact with and activate the protein kinase SOS2 which phosphorylates SOS3 proteins. SOS3–SOS2 interactions recruit SOS2 to the plasma membrane, leading to activation of the downstream target SOS1, an Na^+/H^+ antiporter allowing extrusion of excessive Na^+ from the cytosol (Ji *et al.*, 2013). However, in

the present model, neither of these early linked events, Ca^{2+} increase and $^1O_2$, seemed to be involved in PCD.

On the other hand, Tiron, a scavenger of $O_2^{\cdot-}$, and DPI, an inhibitor of NADPH-oxidase, decreased the NaCl- and sorbitol-induced cell death and the delayed generation of ROS. This indicated that the delayed and more sustained $O_2^{\cdot-}$ generation from NADPH-oxidase activity could play a central role in the death of these cells. Several reports implicate NADPH-oxidase activity in production of ROS in salinity stress, with the ROS resulting in Ca^{2+} influx. In the present model, no effect of DPI on early stress-induced Ca^{2+} increase could be detected (Supplementary Fig. S2 available at *JXB* online), in accordance with the delayed DPI-sensitive ROS generation. However, from the present data, a delayed increase in Ca^{2+} linked to NADPH-oxidase activity cannot be excluded, but this increase in Ca^{2+} should be La^{3+} independent, since La^{3+} could not decrease cell death. It is obvious that sorbitol-induced ROS generation could not depend on Na^+ influx and thus possibly other hyperosmotic-induced events (e.g. NO production) could participate in NADPH-oxidase-dependent ROS generation observed in response to sorbitol and NaCl. However, upon NaCl stress, the delayed ROS generation could be decreased by quinine and verapamil, putative inhibitors of NSCCs (Demidchik *et al.*, 2002*a*, *b*). Even though no definitive molecular candidates have clearly emerged for NSCCs, it seems that various classes of NSCCs could be responsible for influx of Na^+ under salt stress, especially a depolarization-activated class of NSCCs (Demidchik and Maathuis, 2007). A rapid and large depolarization of the BY-2 cells could be recorded upon NaCl addition due to an increase in a current sensitive to quinine and verapamil but also to TEA^+, an inhibitor of K^+ channels known to block some NSCCs (Demidchik *et al.*, 2002*a*). It could also be verified that the accumulation of Na^+ in the cell was decreased upon pre-treatment with quinine or TEA^+, strongly suggesting that NSCCs were responsible for Na^+ influx into BY-2 cells. Moreover, these NSCC blockers were efficient in decreasing the NaCl-induced cell death, highlighting the toxic effect of Na^+ as previously reported (Huh *et al.*, 2002; Affenzeller *et al.*, 2009; Wang *et al.*, 2010). It is further noticeable that La^{3+}, a potent inhibitor of some NSCCs (Demidchick *et al.*, 2002*a*, *b*), was unable to decrease NaCl-induced cell death as well as the delayed ROS generation, whereas the NSCC blockers quinine, verapamil, and TEA^+ failed to decrease the NaCl-induced Ca^{2+} increase (Supplementary Fig. S3 available at JXB online). This indicates that the Ca^{2+} influx was completely dissociated from Na^+ influx through NSCCs. Moreover, sorbitol- and NaCl-induced Ca^{2+} influxes presented the same characteristics and were induced upon hyperpolarization in the case of sorbitol and depolarization in the case of NaCl, suggesting the voltage independence of the transporter involved. Further studies will be needed to determine if putative ligand-activated calcium channels such as cyclic nucleotide-gated channels or glutamate receptors could be responsible for these Ca^{2+} influxes in BY-2 cells; however, a putative candidate could be the calcium regulatory protein annexin (Laohavisit *et al.*, 2013).

Concerning ion flux regulation in response to sorbitol, a hyperpolarization of the BY-2 cells due to the decrease of anion currents was observed, as previously described in different models (Pennarun and Maillot, 1988; Teodoro *et al.*, 1998; Shabala *et al.*, 2000). Although such a regulation of anion current was shown to be involved during PCD induced by $HrpN_{ea}$, a hypersensitive elicitor from *Erwinia amylovora* (Reboutier *et al.* 2005), it did not seem to be involved in sorbitol-induced PCD since bromotetramisole, an activator of anion channels, failed to limit sorbitol-induced PCD (data

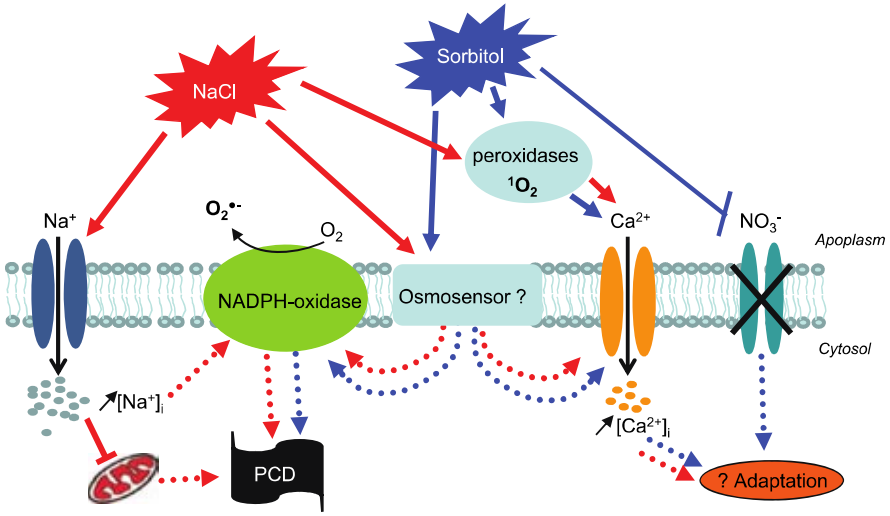

Fig. 8. Possible pathways induced by NaCl and sorbitol leading to cell death of tobacco cells. (This figure is available in colour at *JXB* online.)

not shown), in contrast to the previously observed effect of $HrpN_{ea}$ (Reboutier *et al.*, 2005).

Although ROS-activated outward-rectifying K^+ channels (KORCs) were shown to be involved in salt-induced PCD (Demidchik *et al.*, 2003, 2010), it was not possible to detect rapid activation of KORCs in response to NaCl or sorbitol. However, an activation of KORCs after addition of NaCl cannot be excluded, this current being masked in the large Na^+ current recorded. On the other hand, KORC activation could also be delayed, as was recently reported in response to O_3 (Tran *et al.*, 2013).

Finally, one of the most important differences observed between sorbitol- and NaCl-induced PCD in BY-2 cells was the role of mitochondria. Sorbitol did not affect mitochondrial polarization and even slightly and transiently stimulated its polarization, whereas NaCl induced a large mitochondrial depolarization. This depolarization was probably involved in the formation of the PTP since CsA, an inhibitor of PTP, could reduce the NaCl-induced decrease in $\Delta\Psi_m$ as previously described (Wang *et al.*, 2010) and also the NaCl-induced cell death. This could be related to observation made on *Micrasterias denticulate* (Affenzeller *et al.*, 2009) for which a typical laddering of the DNA was only observed for NaCl-stressed cells but not for sorbitol-treated cells. The endonuclease responsible for this laddering could be released from mitochondria through PTPs (Vianello *et al.*, 2007). It is to be noted that the inhibitors of NSCCs, verapamil, quinine, and TEA^+, were also efficient in limiting the loss in $\Delta\Psi_m$, suggesting a direct role for cytosolic Na^+-induced mitochondrial dysfunction.

As already described in numerous models, in BY-2 cells overlapping responses exist in response to ionic and non-ionic hyperosmotic stress and, among them, a PCD process. However, some overlapping responses such as the early transient $^1O_2$ generation responsible for an influx of Ca^{2+} did not seem to be involved in PCD progress, while other shared responses such as the delayed NADPH-oxidase stimulation were important for these processes (Fig. 8). Specific responses also seemed to be involved in the PCD processes since upon NaCl stress, NADPH-oxidase stimulation was at least partly due to Na^+ influx through NSCCs, which also seemed to be responsible for mitochondrial depolarization not observed after the sorbitol challenge. Further studies will be needed to characterize fully the pathways leading to PCD induced by these hyperosmotic stresses.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Typical anion current recorded in control conditions in BY-2 cells.

Figure S2. NaCl- and sorbitol-induced $[Ca^{2+}]_{cyt}$ increase in aequorin-expressing-tobacco BY-2 cells after a pre-treatment with the inhibitor of NADPH-oxidase DPI (20 µM).

Figure S3. NaCl-induced $[Ca^{2+}]_{cyt}$ increases in aequorin expressing-tobacco BY-2 cells after pre-treatments with the NSCC blockers quinine, verapamil, or TEA^+.

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